EVALUATION OF TRANSGENIC ANTHURIUMS EXPRESSING THE SHIVA-1 GENE ENCODING A SYNTHETIC ANTIMICROBIAL PEPTIDE

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By
TODD M. FUJII

Thesis Committee:

Adelheid Kuehnle, Chairperson
Richard Criley
Anne Alvarez

We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Horticulture

THESIS COMMITTEE

Adelheid R. Kuehle
Chairperson

Richard A. Criley

Anne Alvarez

ABSTRACT

The effect of anthuriums genetically engineered to express a secreted cercropin analogue Shiva-1 on bacterial blight, caused by *Xanthomonas campestris* pv. *dieffenbachiae* and non-target bacteria species was determined. Two cultivars of blight susceptible anthuriums, 'Paradise Pink' (formerly labeled 'Marian Seefurth') and 'Tropic Flame', actively expressing the Shiva-1 transgene were disease-challenged with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachiae* (*Xcd* Lux). Bioluminescence produced by infected leaves was captured on X-ray film enabling the monitoring and quantification of infection. Disease severity as percentage leaf area infected with *Xcd* Lux was compared among replicated transgenic lines and non-transgenic control plants over three months. Two transgenic lines of 'Paradise Pink', MS 1-2 and MS 1-4, displayed enhanced resistance to the anthurium bacterial blight. At the termination of the experiment disease severity of MS 1-2 and MS 1-4 was significantly less ($Pr > F = 0.0001$) than the naturally resistant cultivar 'Kalapana'. One transgenic line of 'Tropic Flame', UH 712 1-16, exhibited enhanced susceptibility to anthurium bacterial blight. Disease severity of UH 712 1-16 was significantly higher ($Pr > F = 0.0038$) than the control line and other transformed lines of the same cultivar. No enhanced resistance was observed among other transgenic UH 712 lines and the control.

In order to understand if biological control strategies are to be used in conjunction with genetic resistance, the effect of transgenic anthuriums producing the Shiva-1 lytic peptide on beneficial bacteria was also studied. Inhibitory concentrations of Shiva-1 against four foliar biocontrol bacteria (BCAs) and *Xcd* were determined by dilution plating after 18 hours exposure to the lytic peptide. Populations were measured in

guttation fluid 6 and 11 days post-inoculation. Two of four BCAs, Gut 3 and Gut 6 were statistically less sensitive to Shiva-1 then *Xcd* ($P < 0.05$); *Xcd* was completely inhibited at 0.5 micromolar. Populations of Gut 3 and Gut 6 increased at 1 micromolar Shiva-1. Transgenic anthuriums, 'Paradise Pink', MS 1-1, MS 1-5 and 'Mauna Kea', MK 1-2, MK 2-6 did not inhibit BCAs more than non-transgenic anthuriums. Biological control with the BCAs, *Sphingomonas chlorophenolica*, *Microbacterium testaceum*, *Brevundimonas vesicularis*, and *Herbaspirillium rubrisubalbicans* is thus compatible with engineered genetic resistance to bacterial blight on anthurium.

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CHAPTER 1

INTRODUCTION

Anthuriums are Hawaii's most important cut flower crop with a wholesale value of $7.4 million in 2000 (National Agriculture Statistics Service Hawaii Department of Agriculture, 2001). In the mid 1980s to early 1990s, Hawaii's anthurium production declined due to an outbreak of disease known as anthurium bacterial blight.

Anthurium blight is caused by the bacterium *Xanthomonas campestris* pv. *dieffenbachiae (Xcd)*. This disease is difficult to control because it can be spread easily by various means such as splashing of rain and/or irrigation, aerosols and contaminated tools and hands. In addition, warm humid conditions help foster this disease. Bacterial blight causes both foliar and systemic symptoms in anthuriums. Classic foliar symptoms include water-soaked spots and yellowing of the leaf margins. Plants infected systemically generally wilt, collapse and eventually die.

No single control measure is effecting in controlling bacterial blight, but the use of disease-free stock plants and stringent sanitation measures are essential in preventing its spread. Tolerant cultivars exist but susceptible cultivars are commonly grown because of strong market demand for their desirable qualities. Today, this disease is less severe than in the 1980s, but its persistence is a continuous threat to Hawaii's anthurium industry.

Researchers at the University of Hawaii, Departments of Tropical Plant and Soil Sciences and Plant and Environmental Protection Sciences have developed two new promising means for controlling of anthurium blight. Genetically engineered anthuriums

producing the Shiva-1 lytic peptide displayed significant delays in the onset of systemic infection of anthurium blight (Kuehnle et al., 1996). Application of a mixture of four species of beneficial bacteria, indigenous to leaves of field-grown anthurium, also suppressed infection of *Xcd* (Fukui et al., 1999a,b). This thesis research continues along the lines of previous studies by screening different anthurium cultivars transgenic for the Shiva-1 lytic peptide for their resistance to bacterial blight. A second objective is to determine if these genetically engineered anthuriums alter the population of the beneficial bacteria and to determine concentrations of Shiva-1 that are lethal to the beneficial bacteria and to *Xcd*.

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CHAPTER 2

LITERATURE REVIEW

2.1 *ANTHURIUM*

The genus *Anthurium* belongs to the family *Araceae* and consists of about 1,000 species (Croat, 1992). Grayum (1990) places *Anthurium* under the subfamily Pothoideae; Bogner and Nicolson (1991) place this genus under the subfamily Lasioideae. *Anthurium* is distributed from northern Mexico to Central America to southern Brazil and the Caribbean Islands (Kamemoto and Kuehnle, 1996).

In 1889, S.M. Damon introduced *Anthurium andraeanum* Linden ex Andre into Hawaii from London (Neal, 1965). By the 1930s and 1940s cultivation of anthuriums by seed was widespread throughout Hawaii. Today, anthurium is Hawaii's most important commercial cut flower crop with most cultivars arising from *A. andraeanum*. In 2000, Hawaii sold about 11.1 million of stems worth $7.4 million farm-gate value (National Agriculture Statistics Service Hawaii Department of Agriculture, 2001). The heart of production is located on the Big Island of Hawaii where areas such as Hilo, Mountain View and Pahoa have temperatures ranging from 55 to 70 °F (13-21 °C) and annual rainfall up to 165 inches (419 cm), providing ideal conditions for growing anthuriums.

In addition to Hawaii, the Netherlands is a major producer of anthuriums. In 1998 anthuriums ranked 13th in total sales among all cut-flower sales sold in the Dutch auctions, with just under 46 million stems sold for a wholesale value of $34.5 million (~29.5 million Euro; International Floriculture Quarterly Report, 2000). Anthurium leaves are also sold at Dutch auctions. In 1998 a total of 12.1 million leaves were sold,

fetching a wholesale value of $2.1 million (~1.8 million Euro; International Floriculture Quarterly Report, 2000). Anthurium cut flowers are produced in many nations as a result of the marketing thrust of the anthurium breeders of the Netherlands. Cultivar improvement through breeding is taking place in areas such as Brazil, the Philippines, and the United States (California and Florida).

Anthuriums are also sold as blooming potted plants. *A. scherzerianum* is a popular potted plant species, and hybrids of miniature species *A. amnicola* and *A. antioquiense* have improved Hawaii's potted plant industry (Kamemoto and Kuehnle, 1996). Potted anthurium breeding has also progressed in Florida using these species. In 2000, Hawaii sold 182,000 potted anthuriums valued at $677,000, an 80% increase in value from 1996 (National Agriculture Statistics Service Hawaii Department of Agriculture, 2001).

2.1.1 Anthurium Bacterial Blight

Anthurium bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae* Lind. Ex Andre (*Xcd*) is considered one of the most devastating diseases for anthurium growers. This disease was first recorded on anthuriums in Brazil in 1960 by C. Robbs (Hayward, 1972). In 1971, Hayward first reported this disease in Hawaii on the island of Kauai (Hayward, 1972). Eventually the blight spread to the islands of Hawaii and Oahu in 1980 (Nishijima et al., 1985). Anthurium blight is also prevalent in the continental United States and in many anthurium producing countries, including the Netherlands (Cooksey, 1985; Mu, 1990; Natural, 1990; Young, 1990; and Sathyanarayana et al., 1998).

An outbreak of anthurium blight in late 1980s became a serious problem statewide for Hawaii (Nishijima et al., 1985). The average loss per farm in 1987 was estimated to be $98,000, accounting for a total industry loss of about $5.5 million (Inouye, 1988; Shehata, et al., 1990). In addition, Hawaii lost its European markets when export production declined and Dutch production and quality improved.

2.1.2 Infection Process and Symptomology

This disease is difficult to control because it is easily spread and latent (symptomless) infections occur within fields. Disease is spread by splashing of rain and/or irrigation, aerosols, and contaminated cutting tools and hands, and introduction of infected planting material (Nishijima, 1988; Alvarez et al., 1994). Diseased plants may harbor the bacteria for more than a year before showing symptoms while serving as a source of inoculum (Norman and Alvarez, 1994). *Xcd* survives mainly in infected plant material and can survive in the soil as a free-living organism for three to six weeks (Natural, 1992).

Xcd infects naturally by entering hydathode pores on leaf margins and through wounds (Hayward, 1972; Sakai et al., 1990). Bacteria entering the hydathodes may be able to move in the guttation fluid to xylem vessels (Sakai et al., 1990). Amino acids in the guttation fluid and xylem sap serve as a food source for the invading bacteria (Sakai, 1990).

Infected anthuriums display two main types of symptoms, foliar and systemic. Symptoms occurring on the leaf or spathe are referred to as foliar symptoms. Initial symptoms are small water-soaked spots, prevalent on the abaxial side of the leaf, and

yellowing of the leaf margins. Infected tissue eventually becomes necrotic and brown. Systemic symptoms occur when the bacteria enter the xylem vessel members. The bacteria spread rapidly, clogging the vascular system and preventing translocation of water and nutrients. This eventually causes the plant to wilt and collapse. Disease symptoms are severe in wet, warm conditions, with optimal temperature for disease development at 30 °C (Nishijima et al., 1985; Alvarez et al., 1990; Fukui et al., 1999a).

2.1.3 Susceptibility to Blight among Cultivars

A limited study shows that susceptibility to foliar and systemic blight varies among commercial cultivars (Table 2-1). 'Marian Seefurth' (H 33) is susceptible to both foliar and systemic infection phases, while 'Kalapana' (UH 1016) is resistant in both phases (Fukui et al., 1998). Other cultivars are resistant to one infection phase while susceptible to the other. For example, 'ARCS' (UH 1068) is severely affected by foliar infection but resistant to systemic blight while 'Tropic Mist' (UH 780) easily succumbs to systemic infection but shows little foliar infection (Fukui et al., 1998). It is not fully understood how the means of resistance in foliar infection relate to that of systemic infection.

2.1.4 Current Control Measures

There is no single measure effective in controlling or eradicating the disease. Current controls include strict sanitation measures, "sensible" culture practices (preventing splashing, disinfestation of tools) and the use of disease-free propagation stock. A number of chemicals and antibiotics have been tested to control anthurium

Table 2-1. Susceptibility of University of Hawaii anthurium cultivars to bacterial blight (Fukui et al., 1998).

Cultivar	Foliar infection	Systemic infection
ARCS	S	VR
Alii	VR	S
Kalapana	R	VR
Marian Seefurth	VS	VS
Nitta*	VR	S
Pink Elf	VS	S
Tropic Mist	R	VS

S, susceptible; VS, very susceptible;
R, resistant; VR, very resistant
* not University of Hawaii cultivar

blight (Nishijima, 1988; Alvarez et al., 1989; Nishijima et al., 1991). Disease incidence may be reduced by 40-50% by application of fosetyl-aluminum and/or agribrom but active infections still provide inoculum for secondary spread, so treatments must be frequent and are not economically feasible. Attempts to control the disease with copper compounds such as copper hydroxide and copper sulfate were unsuccessful (Nishijima, 1988). *Xcd* tolerated these compounds and the compounds were phytotoxic to the anthuriums (Nishijima, 1988). Antibiotics are not recommended for routine control because the bacteria develop streptomycin resistance in about two years (Nishijima, 1988).

The key method of control relies on the combination of using disease-free planting stock and strict sanitation measures. Sanitation alone fails to control the disease because the source of the disease could be from latently infected propagation material in addition to infected plants already growing in the field. When the pathogen is introduced into fields though symptomless propagative stock, severe disease occur after three years (Norman and Alvarez, 1996). Thus efforts have been made to propagate anthuriums in vitro for stock material and to certify them as disease-free by triple indexing (Tanabe et al., 1994a; Tanabe et al., 1994b; and Tanabe et al., 1995).

In addition to these control measures, another approach used for controlling anthurium blight is through breeding and cultivating resistant cultivars. By breeding with the blight resistant species *A. antioquiense*, Kamemoto attempted to transfer its apparent systemic resistance to the cultivated *A. andraeanum* (Kamemoto et al., 1990). F1 hybrids displayed high degrees of resistance although the flowers had poor horticultural characteristics. Therefore, backcrossing was necessary to obtain resistant plants with

improved the flowers. In spite of these breeding efforts, the genetic basis of blight resistance is still not fully understood.

2.1.5 Biological Control of *Xanthomonas campestris* pv. *dieffenbachiae*

The use of bacteria to control fungal diseases has been studied intensely (reviewed by Shodo, 2000). *Agrobacterium, Pseudomonas, Bacillus, Alcaligenes,* and *Streptomyces* have been reported as biocontrol agents for fungal diseases. However, little has been published on the use of bacterial control against bacterial diseases of plants. Anuratha and Gnanamanickam (1990) reported on the biological control of bacterial wilt caused by *Pseudomonas solanacearum* with antagonistic bacteria species *P. fluorescens* and *Bacillus* spp.

Fernandez and colleagues examined the use of endophytic and epiphytic bacteria from different aroids for biological control for anthurium blight (Fernandez et al., 1988, 1989, 1990, 1991.) Bacteria isolated from vascular tissue and leaf surfaces of *A. andraeanum* and other aroids were screened in vitro for their antagonistic effects against *Xcd*. Microorganisms that were antagonistic to *Xcd* in vitro were applied to the cultivar 'Marian Seefurth' as a protective spray under field conditions. None of the potential antagonists individually or in combination reduced foliar and systemic blight infection in field conditions. Thus, the experiments were terminated.

Along the same lines, Fukui et al. (1999b,c) explored the role of bacteria, indigenous to anthurium guttation fluid, as a biological control of anthurium bacterial blight in vitro and in greenhouse conditions. Growth and survival of *Xcd* in vitro was suppressed by a mixture of four bacteria species originally identified as *Pseudomonas*

paucimonilis, Brevundimonas vesicularis, Microbacterium sp. and another *Pseudomonas* sp (Fukui et al., 1999c). Using 16-S rDNA analysis, the bacteria were later identified as *Sphingomonas chlorophenolica, Microbacterium testaceum, Brevundimonas vesicularis,* and *Herbaspirillum rubrisubalbicans* (Alvarez et al., 2001). This mixture was more effective than any individual species. Several anthurium cultivars were also protected from invasion of *Xcd* through hydathodes and wounds when their leaves were inoculated with the bacterial mixture at concentrations ranging from 2.0 X 10^8 to 3.0 X 10^8 CFU/ml (Fukui et al., 1999b). While the mechanism of *Xcd* suppression by the four strains of bacteria is not known, competition for organic nutrients is thought to be involved (Fukui et al., 1999b).

2.2 GENETIC ENGINEERING FOR DISEASE RESISTANCE

2.2.1 Antimicrobial Proteins and Peptides Isolated from *Hyalophora cecropia*

Antibacterial proteins and peptides are a component of the immune defense system of prokaryotic and eukaryotic organisms. A host of these peptides have been isolated, mostly from insects (review by Bulet et al., 1999; Table 2-2). It was proposed that genes for these peptides with antibacterial properties may enhance disease resistance in horticulturally valued plants (Jaynes et al., 1987; Casteels et al., 1989).

Cecropins are a family of small basic peptides isolated from the hemolymph of the giant silk moth, *Hyalophora cecropia.* These peptides, which are produced in response to bacterial infection, are 31 to 39 amino acids in length and comprise three major forms (Mills and Hammerschlag, 1993). Cecropins interact with bacterial

Table 2-2. Antibacterial proteins and peptides derived from insects.

Protein/Peptide	Origin	Reference
Abaecin	*Apis mellifera*	Casteels et al., 1990
Apidaecins	*Apis mellifera*	Casteels et al., 1993
Cecropins	*Hyalophora cecropia*	Bowman et al., 1991
Defensin	*Calliphora vicina*	Bulet et al., 1999
	Esristalis tenax	Bulet et al., 1999
	Drosophila melanogaster	Bulet et al., 1999
	Anopheles gambiae	Bulet et al., 1999
	Limnephilus stigma	Bulet et al., 1999
	Apis mellifera	Bulet et al., 1999
	Bombus pascuorum	Bulet et al., 1999
	Formica rufa	Bulet et al., 1999
	Allomyrina dichotoma	Bulet et al., 1999
	Pyrrhocoris apterus	Bulet et al., 1999
	Palomena prasina	Bulet et al., 1999
	Notonectes glauca	Bulet et al., 1999
	Chrysopa perla	Bulet et al., 1999
	Aeschna cyanea	Bulet et al., 1999
	Leiurus quinquestriatus	Bulet et al., 1999
	Androctonus australis	Bulet et al., 1999
	Mytilus galloprovinsialis	Bulet et al., 1999
Defensin A	*Phormia terranovae*	Bulet et al., 1999
	Aedes aegypti	Bulet et al., 1999
	Zophobas atratus	Bulet et al., 1999
Defensin B	*Phormia terranovae*	Bulet et al., 1999
	Aedes aegypti	Bulet et al., 1999
Defensin 1	*Podisus maculiventris*	Bulet et al., 1999
	Mytilus edulis	Bulet et al., 1999
Defensin 2	*Podisus maculiventris*	Bulet et al., 1999
	Mytilus edulis	Bulet et al., 1999

Table 2-2 (cont.). Antibacterial proteins and peptides derived from insects

Protein/Peptide	Origin	Reference
Drosomycin	*D. melanogaster*	Fehlbaum et al., 1994
Holotricin 1	*Holotrichia diomphalia*	Fehlbaum et al., 1994
Holotricin 1A	*Holotrichia diomphalia*	Fehlbaum et al., 1994
Holotricin 1B	*Holotrichia diomphalia*	Fehlbaum et al., 1994
Melittin	*Apis melifera*	Steiner et al., 1981
Sapecin A	*Sarcophaga peregrina*	Steiner et al., 1981
Sapecin C	*Sarcophaga peregrina*	Steiner et al., 1981
Tenicin 1	*Allomyrina dichotoma*	Steiner et al., 1981
Thanatin	*Podisus maculiventris*	Fehlbaum et al., 1996

membranes as amphipathic molecules by forming channels and eventually disrupt the membrane sufficiently to cause the cell to lyse. Cecropins bear antibacterial properties against both gram-negative and gram-positive bacteria (Huang et al., 1997).

SB-37 and Shiva-1 are synthetically produced analogs of the natural cecropins, and both are 38 amino acids in length. SB-37's amino acid sequence is 95% homologous to cecropin B and Shiva-1 shares 46% homology (Jaynes et al., 1993). These cecropin-like peptides possess similar or increased antibacterial activity relative to natural cecropins.

Attacins are another group of lytic proteins isolated from the giant silk moth that are around 20 kD. The bactericidal effects of attacins have been recognized in vitro on gram negative bacteria (Hultmark, 1993). Unlike cecropins, the mode of action of attacin is not fully understood (Düring, 1996; Mourgues et al., 1998). However, some believe attacins inhibit growth of gram negative bacteria by increasing the permeability of the outer membrane, allowing the passage of hydrophobic and hydrophilic substances (Carlsson et al., 1991; Carlsson et al., 1998).

2.2.2 Antimicrobial Proteins and Peptides Isolated from Amphibians

Like the insects, amphibians represent a large source of antimicrobial proteins and peptides (Table 2-3). According to Rao (1995), Csordas and Michl (1970) were the first to report this finding, isolating a 24 amino acid residue antibiotic peptide (bombinin) from the skin of the European toad, *Bombina variegata*. Other antimicrobial peptides include magainin and magainin-like peptides isolated from skin secretions of the Asian toad, *Bombina orientalis*, and frog, *Xenopus laevis*, respectively (Zasloff, 1987; Gibson et

Table 2-3: Antimicrobial proteins and peptides derived from amphibians.

Protein/Peptide	Origin	Pathogen	Reference
Alamethicin	*Trichoderma viride* (fungus)	antibacterial, antifungal	Bechinger, 1997
Bombinin	*Bombina variegata*	antibacterial, antifungal	Csordas and Michl, 1970
Bombininlike	*Bombina orientalis*	antibacterial, antifungal	Gibson et al., 1991
Brevinin 1	*Rana brevipoda porsa*	antibacterial, antifungal	Morikawa et al., 1992
Brevinin 2	*Rana brevipoda porsa*	antibacterial, antifungal	Morikawa et al., 1992
Brevinin 1E	*Rana esculenta*	antibacterial, antifungal	Simmacco et al., 1993
Brevinin 2E	*Rana esculenta*	antibacterial, antifungal	Simmacco et al., 1993
Dermaseptin	*Phyllomedusa sauvagii*	antibacterial, antifungal	Mor et al., 1991
Magainins	*Xenopus laevis*	antibacterial, -fungal, -viral	Zasloff, 1987
Nisin	bacteria	antibacterial (gram-pos.)	Hurst and Collins-Thompson, 1970
PGQ	*Xenopus laevis*	antibacterial, antifungal	Moore et al., 1991
Ranalexin	*Rana catasbeiana*	antibacterial, antifungal	Clark et al., 1994

al., 1991). The 24 amino acid residue magainins exhibit bactericidal, fungicidal, and virucidal activities (Zasloff, 1987; Rao, 1995). The exact mechanism of antimicrobial activity of magainins is not defined but their amphipathic α-helical structure is thought to disrupt the integrity of phospholipid bilayers, i.e., they are membranolytic.

2.2.3 Antimicrobial Proteins and Peptides Isolated from Plants

Antimicrobial proteins have also been isolated from plants (reviewed by Broekaert, 1995; Rao, 1995; Table 2-4). These peptides inhibit the growth of a large range of fungi by reducing and slowing hyphael elongation (Broekaert et al., 1995). Most of these peptides are ineffective against bacteria. Thionins are a family of peptides that are 37-45 amino acids long found in the leaf and seed tissues of barley and wheat (Bohlmann and Apel, 1991). In vitro they posses antimicrobial properties against bacteria, yeast and fungi and are thought to be a component of the plant defense mechanism (Bohlmann, 1994). MBP-1 is a 33-residue peptide isolated from maize kernels (Duvick et al., 1992). It has both fungicidal and bactericidal properties against maize pathogens. Ac-AMP1 is a peptide similar in size from the seeds of *Amaranthus caudatus* (Broekaert et al. 1992). This 29-residue peptide exhibits activity against gram-positive bacteria and some plant pathogenic fungi. Geraniums transformed with a gene encoding the *Allium cepa* antimicrobial protein 1 (Ace-AMP1) isolated from onion seeds were shown to have increased resistance to *Botrytis cinerea* leaf infection (Bi et al., 1999).

Table 2-4. Antimicrobial proteins and peptides derived from plants and other organisms.

Protein/Peptide	Origin	Activity	Reference
Ac-AMP1	*Amaranthus caudatus*	antibacterial, antifungal	Broekaert et al. 1992
Ac-AMP2	*Amaranthus caudatus*	antibacterial, antifungal	Broekaert et al. 1992
Ace-AMP1	*Allium cepa*	antifungal	Bi et al. 1999
Ah-AMP1	*Aesculus hippocastanum*	antifungal	Osborn et al., 1995
Ct-AMP1	*Clitoria ternatea*	antibacterial, antifungal	Osborn et al., 1995
Dm-AMP1	*Dahlia merckii*	antifungal	Osborn et al., 1995
FST	*Nicotiana tabacum*	antifungal	Gu et al., 1992
Hs-AFP1	*Heuchera sanguinea*	antifungal	Osborn et al., 1995
MBP-1	*Zea maize*	antibacterial, antifungal	Duvick et al. 1992
p322	*Solanum tubersosum*	antifungal	Stiekema et al., 1988
pI230	*Pisum sativum*	antifungal	Chiang and Hadwiger, 1991
PPT	*Petunia inflata*	antifungal	Karunanandaa et al., 1994
pSAS10	*Vigna unguiculata*	antifungal	Ishibashi et al., 1990
Rs-AFP1	*Raphanus sativus*	antifungal	Terras et al., 1992, 1995

Protein/Peptide	Origin	Activity	Reference
Rs-AFP2	*Raphanus sativus*	antifungal	Terras et al., 1992, 1995
Siα2	*Sorghum bicolor*	antifungal	Bloch and Richardson, 1991; Nitti et al., 1995
Thionins	barley, wheat	antibacterial, -fungal, -yeast	Bohlmann and Apel, 1991
γ1-P	*Triticum aestivum*	antibacterial, -fungal, -yeast	Collila et al., 1990
γ1-H	*Hordeum vulgare*	antibacterial, -fungal, -yeast	Mendez et al., 1990
Epidermin	bacteria	antibacterial (gram-pos.)	Rao, 1995
Subtilin	bacteria	antibacterial (gram-pos.)	Rao, 1995
Tachyplesin	*Tachypleus tridentatus*	antifungal	Nakamura et al. 1988
no name	*Aspergillus giganteus*	antifungal	Nakaya et al. 1990

2.2.4 Genetic engineering studies

The first genetically engineered transgenic plants, containing prokaryotic marker genes were produced more than twenty years ago (Otten et al., 1981). Subsequently, genes from insects, plants, and other organisms have been engineered into plants to enhance disease resistance, herbicide tolerance, and crop yield among other horticulturally useful traits.

Much effort has been focused on isolating antimicrobial genes and improving gene expression to obtain significant levels of disease resistance in plants. Some success has been achieved in improving disease resistance by introducing genes for antibacterial peptides (Table 2-5). Only recently have several reports appeared that demonstrate utility of this approach, building on research that began in the late 1980s (Carlson et al., 1998; Arce et al., 1999; Reynoird et al., 1999). However, transgenic plants displaying resistance to bacterial or fungal disease are yet to be commercially produced (Salmeron and Vernoorij, 1998).

Jaynes et al. (1993) produced transgenic tobacco seedlings exhibiting delayed symptom production and reduced disease severity and mortality after inoculation with *Pseudomonas solanacearum*. Tobacco leaves were transformed by *Agrobacterium tumefaciens* carrying genes encoding the cecropin-like synthetic peptides SB-37 and Shiva-1. Nucleic acid base sequences of SB-37 and Shiva-1 were synthesized and cloned into the binary vector pBI121 under control of a wound inducible plant promoter, proteinase inhibitor II (PiII). Expression of the peptide genes were determined at the RNA level using Northern analysis. No resistance was observed in planta between the

Table 2-5. Examples of improved disease resistance in plants genetically engineered for expression of antimicrobial proteins.

Foreign Gene	Origin	Transgenic Plant	Target Pathogen	Reference
Ace-AMP1	*Allium cepa*	geranium (*Pelargonium sp.*)	*Botrytis cinerea*	Bi et al., 1999
Attacin E	*Hyalophora cecropia*	pear (*Pyrus communis*)	*Erwinia amylovora*	Reynoird et al., 1999
		apple (*Malus domestica*)	*Erwinia amylovora*	Norelli et al., 1994
		potato (*Solanum tuberosum*)	*Erwinia carotovora*	Arce et al., 1999
D4E1	synthetic	tobacco (*Nicotiana tabacum*)	*Colletotrichum destructivum*	Cary et al., 2000
Glucose oxidase	*Aspergillus niger*	potato (*Solanum tuberosum*)	*Erwinia carotovora* ssp. *carotovora*	During et al., 1996
			Phytophthora infestans	During et al., 1996
Lactoferrin	human	tobacco (*Nicotiana tabacum*)	*Ralstonia solanacearum*	Zhang et al., 1998
Lysozymes	T4- bacteriophage	potato (*Solanum tuberosum*)	*Erwinia carotovora atroseptica*	During et al., 1993
	human	tobacco (*Nicotiana tabacum*)	*Pseudomonas syringae* pv. *tabaci*	Nakajima et al., 1997
MB-39	cecropin-like peptide	tobacco (*Nicotiana tabacum*)	*Pseudomonas syringae* pv. *tabaci*	Huang et al., 1997
Shiva-1	cecropin-like peptide	tobacco (*Nicotiana tabacum*)	*Pseudomonas solanacearum*	Jaynes et al., 1993
Tachyplesin	horseshoe crab	potato (*Solanum tuberosum*)	*Erwinia carotovora*	Allefs et al., 1996
Thionin	barley	tobacco (*Nicotiana tabacum*)	*Pseudomonas syringae* pv. *tabaci*	Carmona et al., 1993
			Pseudomonas syringae pv. *syringae*	Carmona et al., 1993

control plants and plants producing SB-37, only Shiva-1 transformed plants showed enhanced resistance to *P. solanacearum*.

Huang and colleagues conferred resistance to *Pseudomonas syringae* pv. *tabaci* in tobacco transgenic for cecropin MB39 (Huang et al., 1997). In their approach, a gene cassette consisting of a protein secretory sequence from barley α-amylase connected to the MB39 coding sequence and PiII promoter and terminator was introduced into tobacco by *Agrobacterium*-mediated transformation. Polymerase chain reaction (PCR) amplification confirmed the presence of MB39 in the plant cells. Plants were challenged by infiltrating leaves with five different inoculum concentrations of the pathogen. While control plants showed visible symptoms at all five levels, plants transgenic for the MB39 sequence displayed necrosis only in leaves infiltrated at the two highest levels. Two transgenic lines exhibiting high levels of disease resistance were selected for further analysis. Western blot assays confirmed MB39 expression in these two lines. Secretion of the peptide into the intercellular spaces was not determined.

In a similar study, tobacco plants transgenic for synthetic cecropins failed to provide resistance to *P. syringae* pv. *tabaci* (Hightower et al., 1994). Degradation of the peptides by intercellular fluids is thought to account for the lack of resistance (Mills et al., 1994; Florack et al., 1995; Mourgues et al., 1998).

Norelli et al. (1994) used the attacin E gene in their attempt to achieve resistance in Malling 26 apple rootstocks to fire blight caused by *Erwinia amylovora*. Transformation was mediated by *Agrobacterium* using the pLDB15 plasmid containing the gene encoding the lytic protein attacin E. Southern analysis confirmed the integration of the attacin E gene into the genome, and the presence of attacin E mRNA was

confirmed by Northern analysis. Transgenic plants showed increased resistance to infection by *E. amylovora*, with less lesions in comparison to control plants. Transgenic plants inoculated in vitro displayed higher LD_{50} values than inoculated untransformed plants of the same cultivar. Similar plants were shown to produce the attacin protein by immunoblot assay (Ko et al., 1999). Ko's group observed a positive correlation between the attacin content and disease resistance among transgenic 'Galaxy' apples with attacin concentration levels ranging from 0 to 0.04 µg/ml (Ko et al., 2000).

Pear plants transformed with attacin E also showed increased resistance to fire blight, *Erwinia amylovora,* in vitro (Reynoird et al., 1999). RNA was extracted and expression levels were determined by Northern analysis and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Western analysis detected expression of the attacin E peptide in the transformed plants. Eleven transgenic lines were obtained and six lines showed improved resistance over controls (decreased progression of necrosis).

Arce et al. (1999) introduced disease resistance to blackleg and soft rot caused by *Erwinia carotovora atroseptica* in potato plants with genes encoding attacin and SB-37. Transformation was accomplished by infecting internodes with *A. tumefaciens* carrying the binary vector pBIAtt and pBISB-37. Presence and expression of attacin and SB-37 were detected by PCR analysis and Northern analysis, respectively. For the SB-37 clones, 18.9% (7 of 37 clones) and 22.6% (7 of 31 clones) showed reduced disease symptoms to blackleg and soft rot, respectively. For the attacin clones evaluated, 15.4% (4 of 26 clones) and 20% (7 of 35 clones) showed reduced disease severity for blackleg and soft rot, respectively. Resistance to blackleg was determined by a "disease

coefficient" calculated as the disease index of control plants divided by the disease index of the transgenic plants. Soft rot resistance was determined by evaluating the appearance of rot symptoms and the sprouting capability of tubers from transformed clones after bacteria inoculation.

In a recent study, tobacco plants transformed with another synthetic antimicrobial peptide, D4E1, displayed resistance to the fungal pathogen *Colletotrichum destructivum* (Cary et al., 2000). The D4E1 peptide was encoded by a gene under the control of an enhanced cauliflower mosaic virus 35S promoter. Transgenic tobacco plants were obtained by using *A. tumefaciens*-mediated leaf disk transformation. Transformation was determined by PCR and Southern hybridization, and expression of the D4E1 gene was demonstrated through RT-PCR.

Studies also have been initiated to determine whether antibacterial lytic peptides have toxic effects on the plant (Nordeen et al., 1992; Mills and Hammerschlag, 1993; Mills et al., 1994). It was determined that peach mesophyll cells and intact leaf tissue are relatively tolerant to cecropin B (lethal concentration between 25 and 50 μM) compared to plant pathogens such as *P. syringae* pv. *syringae* and *Xanthomonas campestris* pv. *pruni* that have lethal concentration between 0.1-0.5 μM (Mills and Hammerschlag, 1993; Mills et al., 1994). Based on these data, scientists concluded transgenic peach trees could produce and accumulate enough cecropin to control *P. syringae* pv. *syringae* while not experiencing cecropin-induced damage in the leaves (Mills et al., 1994).

2.2.5 Genetic Engineering of *Anthurium* for Disease Resistance

Chen (1993) was the first to report on genetic engineering of anthurium for bacterial disease resistance against *Xcd*. Laminae and internodes of University of Hawaii cultivars 'Rudolph' (UH 965) and UH 1060 were transformed by *Agrobacterium tumefaciens* with the antibacterial genes attacin, T4 lysozyme, P13, and Shiva-1. The antibacterial genes were put in the *Hind* III site of the pBI121 vector under control of the double CaMV35S promoter or a wound-inducible promoter potato proteinase inhibitor II (Ca2Att, Ca2T4, Ca2P13, WIAtt and WIShiva). Plants attained from internodes and lamina transformed with the pCa2Att and pCa2P13 plasmids were determined to be transgenic based on kanamycin resistance, PCR amplification of NPTII marker gene and the antibacterial gene and Western blot detection of the protein (attacin only). Rudolph plantlets transgenic for attacin were inoculated with *Xcd* and grown in growth chambers. The majority of the transformed plants showed resistance (no symptoms or mild symptoms). Transgenic plants also had fewer bacteria counts after re-isolation than untransformed control plants.

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CHAPTER 3

DISEASE CHALLENGE OF ANTHURIUMS TRANSGENIC FOR THE SHIVA-1 PEPTIDE WITH A BIOLUMINESCENT STRAIN OF *XANTHOMONAS CAMPESTRIS* PV. *DIEFFENBACHIAE*

3.1 INTRODUCTION

Anthurium hybrids are Hawaii's most important commercial cut flower crop. In 2000, about 11.1 million stems were sold worth $7.4 million (National Agriculture Statistics Service Hawaii Department of Agriculture, 2001). Due to the persistence of bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae* (*Xcd*), this disease is a continuous threat to Hawaii's anthurium industry. In an effort to control this disease, we have genetically engineered anthurium to express a secreted lytic peptide known as Shiva-1.

F.C. Chen (1993) was the first to successfully transform anthuriums with antimicrobial genes and obtain anthuriums resistant to *Xanthomonas campestris* pv. *dieffenbachiae* (*Xcd*). Two-month-old plantlets were challenged by applying bacteria to freshly cut petioles. The majority of transgenic plants (8/13) showed resistance or delayed disease symptom development while control non-transgenic plants (8/9) were highly susceptible. The objective of this follow-on experiment was to screen in replicate different cultivars of mature anthuriums, shown to be transgenic for Shiva-1 at the DNA and RNA and protein levels, using modified disease assessment tools. Transgenic anthuriums were disease-challenged on two separate occasions by spray inoculation on January 30, 2001 and June 28, 2001. In both experiments plants were monitored for resistance over a period of three months. A bioluminescent strain of *Xanthomonas*

campestris pv. *dieffenbachiae* (*Xcd* Lux) was used as inoculum enabling the internal monitoring and quantification of infection by exposing leaves to X-ray film.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material

Experiment I. Two cultivars of transgenic anthuriums were tested in the first experiment. Independent transformants were represented by five transgenic lines of 'Marian Seefurth' (MS 1-1, MS 1-2, MS 1-4, MS 1-5 and MS 1-6) and eight lines of transgenic 'Tropic Flame' (UH 712 1-1, UH 712 1-4, UH 712 1-8, UH 712 1-9, UH 712 1-11, UH 712 1-13, UH 712 1-15, and UH 712 1-16). Non-transformed lines of both cultivars served as controls. Plants transgenic for the Shiva-1 lytic peptide were produced by N. Sugii in August 1994 as described by Chen and Kuehnle (1996) using the pBPRS1 plasmid containing the pathogenesis-related protein 1b secretion signal. Shiva-1 was previously determined by PCR and ELISA to be present in the original mother stock of transformant lines (A. Kuehnle and N. Sugii, unpublished) and subsequently confirmed in a sample of the clonal population of the plants used in these experiments by reverse transcription polymerase chain reaction (RT-PCR; Table 3-1; A. Kuehnle and K. Chuang, unpublished) and Northern analysis (A. Kuehnle, S. Schwartz, and R. Mudalige, unpublished).

Plants of 'Rudolph', blight susceptible, and 'Kalapana', blight tolerant, were included to serve as references for disease severity. Each transgenic, control, and reference check line consisted of six plant replicates.

Table 3-1. PCR, ELISA, and RT-PCR status of transformant lines used in bacterial blight challenge.

Cultivar & line no.	7/96 PCR Shiva-1	7/96 ELISA	5/01 PCR NPT II	6/01 PCR Shiva-1	6/01 RT-PCR
UH 712 control	no test	-	-	-	-
UH712 1-1	no test	+	+	+	+
UH712 1-4	+/ -	+	+	+	+ weak
UH712 1-8	no test	+	+	+	-
UH712 1-9	no test	+	+	+	+
UH712 1-11	no test	+	+	+	no test
UH712 1-13	no test	+	+	+	+
UH712 1-15	no test	+	+	+	+ weak
UH712 1-16	no test	+	+	+	no test
MS control	-	-	-	-	-
MS 1-1	- /+	+	+	+	+ weak
MS 1-2	+	+	+	+	+
MS 1-4	-	+	+	+	no test
MS 1-5	-	+	+	+	no test
MS 1-6	-	+	+	+	-

'Marian Seefurth' is a standard heart-shaped, pink anthurium very susceptible to bacterial blight (Fig. 3-1a). 'Tropic Flame' produces tulip-shaped, red flowers (Fig. 3-1b). It is a seedling selection from a cross between 'Calypso' (A195) and UH 589. 'Tropic Flame' is susceptible to bacterial blight and is not as tolerant to the systemic phase of the blight as its parent 'Calypso'. 'Rudolph' has large white flowers with a red spadix and is also susceptible to blight (Fig. 3-1c). 'Kalapana' is a small to medium red obake type anthurium that is resistant to bacterial blight (Fig. 3-1d). It is a selection from a cross between 'Diamond Jubilee' and 'Paradise Pink'. The latter is an offspring between 'DeWeese' and 'Marian Seefurth' and is susceptible to blight (Kamemoto and Kuehnle, 1996).

Experiment II. Based on the results of Experiment I, MS 1-2, MS 1-4, UH 712 1-1, UH 712 1-9, and UH 712 1-16 were selected for further analysis in a second bacterial challenge. 'Rudolph' and 'Kalapana' were also included. Replications were increased to nine to ten plants per line. Non-transgenic 'Marian Seefurth' plants were not available for Experiment II and therefore substituted with 'Rudolph' which has a similar susceptibility response.

Transgenic lines, control and check plants were micropropagated over a 12-month period in Magenta GA-7 Vessels (Fig. 3-2a) on a Murashige and Skoog (MS) medium modified to consist of ½ MS major salts, 15% coconut water and 2% sucrose (Appendix A). Plants were grown in a culture room at 25°C under a 16-hour photoperiod. Stage 3 plantlets were transferred into community pots containing shredded tree fern fiber (Fig. 3-2b). Once acclimated, the plants were transplanted into flats containing a 3:1 composted redwood bark to perlite (fine grade) planting medium (Fig. 3-2c). Plants in

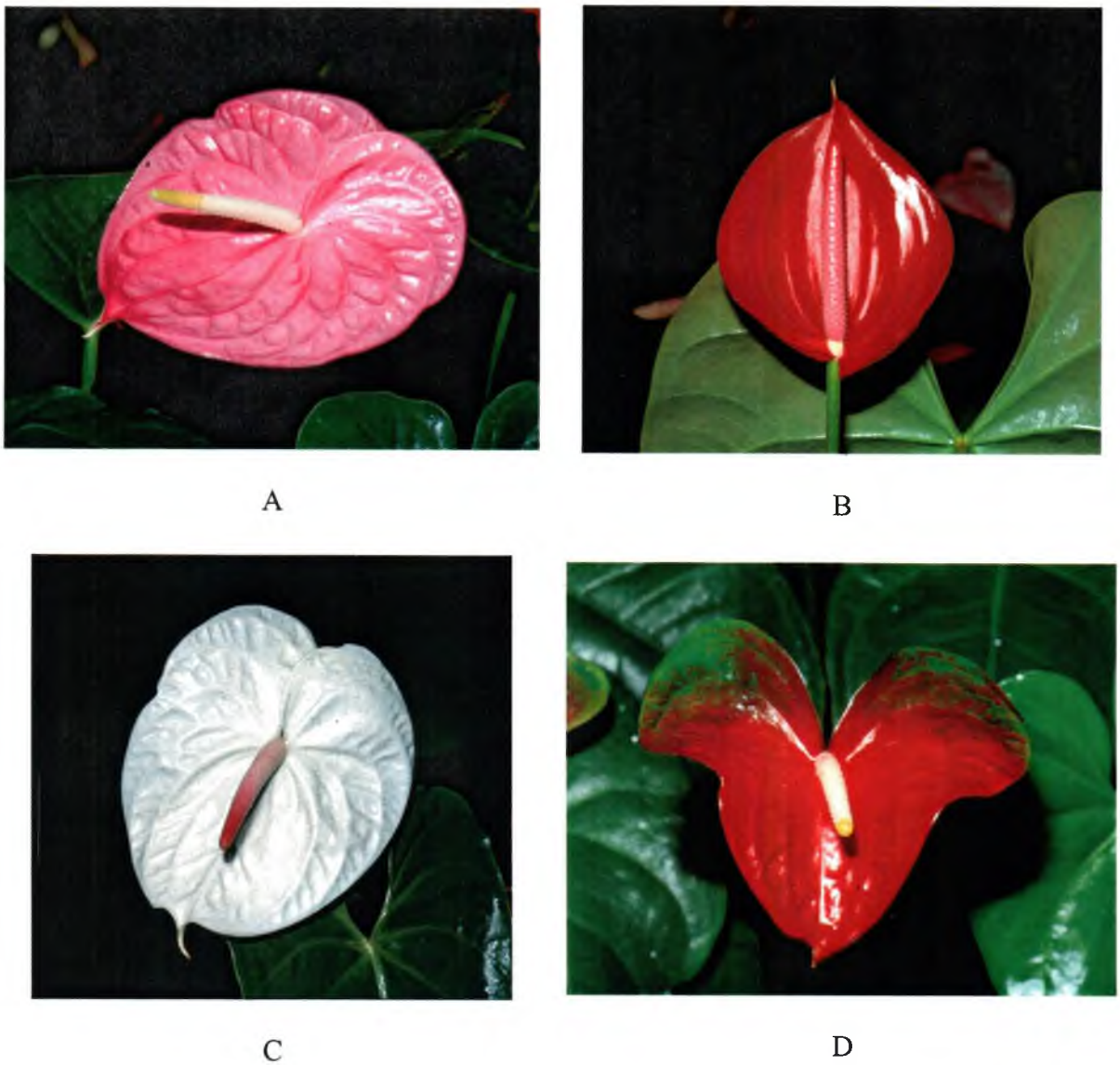


Fig. 3-1. Inflorescences of University of Hawaii anthurium cultivars. (A) 'Marian Seefurth' (H 33). (B) 'Tropic Flame' (UH 712). (C) 'Rudolph' (UH 965). (D) 'Kalapana' (UH 1016). Photographs courtesy of H. Kamemoto.

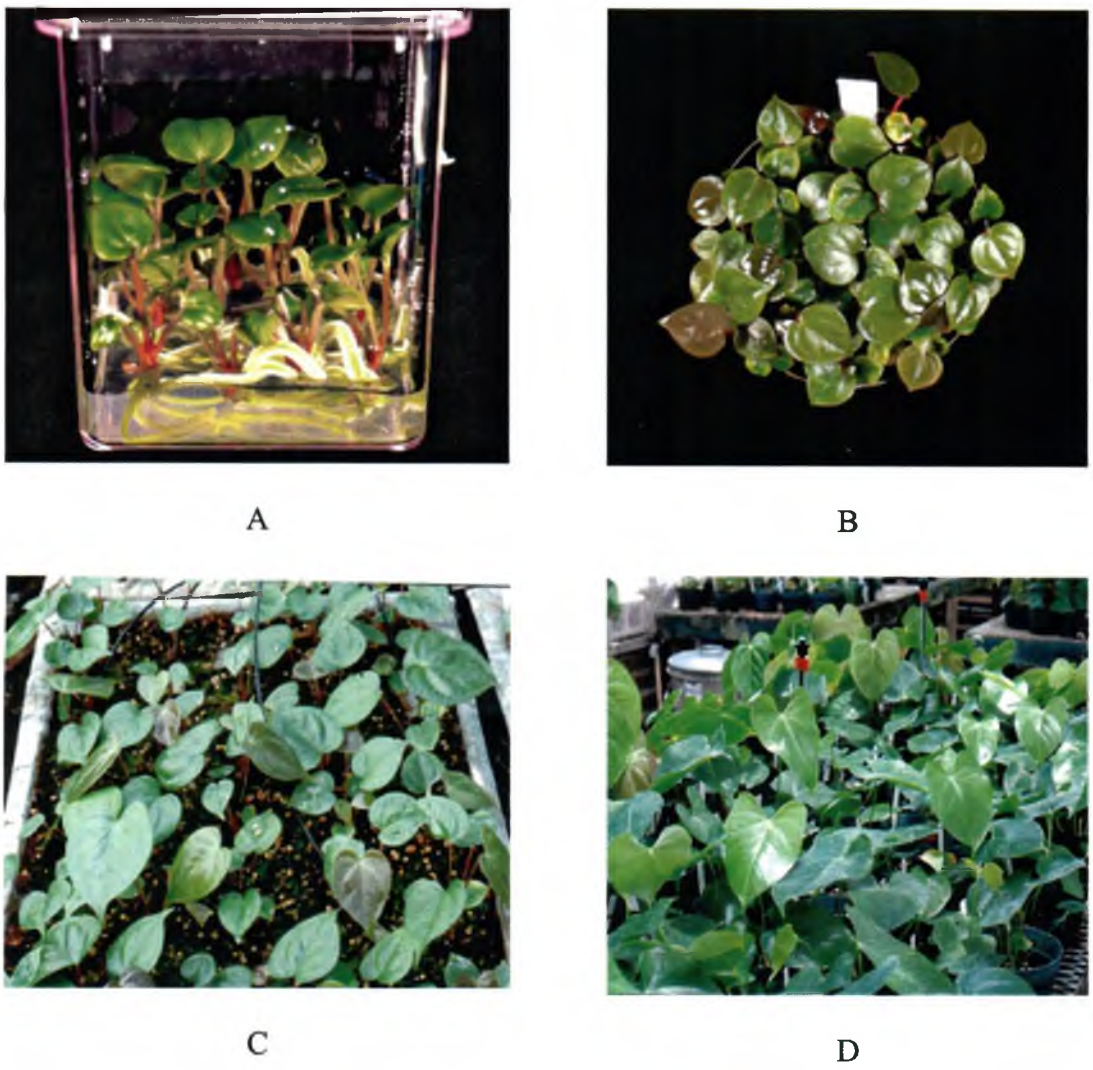


Fig. 3-2. Stages of anthurium propagation. (A) Tissue cultured, plantlets derived from axillary buds. (B) Community pot. (C) Anthuriums in flats. (D) Individual plants in 5" pots.

flats were foliar fed with Miracle-Gro 15-30-15 every two weeks and grown for approximately six months. Anthuriums were transplanted from flats into 5" pots in a 3:1 composted redwood bark to perlite (coarse grade) mixture and fertilized with osmocote (16-16-16; Fig. 3-2d). All plants were grown over an 18-month period in 5" pots in a glasshouse at the University of Hawaii Magoon Facilities in Manoa prior to inoculation.

3.2.2 Bacterial strain

A bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachiae* (*Xcd* Lux) was obtained from A. Alvarez, Department of Plant and Environmental Protection Sciences, and used to examine the progression of leaf infection and to determine disease severity. Light produced by bacteria was detected with X-ray film and infection quantified by the amount of bioluminescence recorded on film (Fukui et al., 1998).

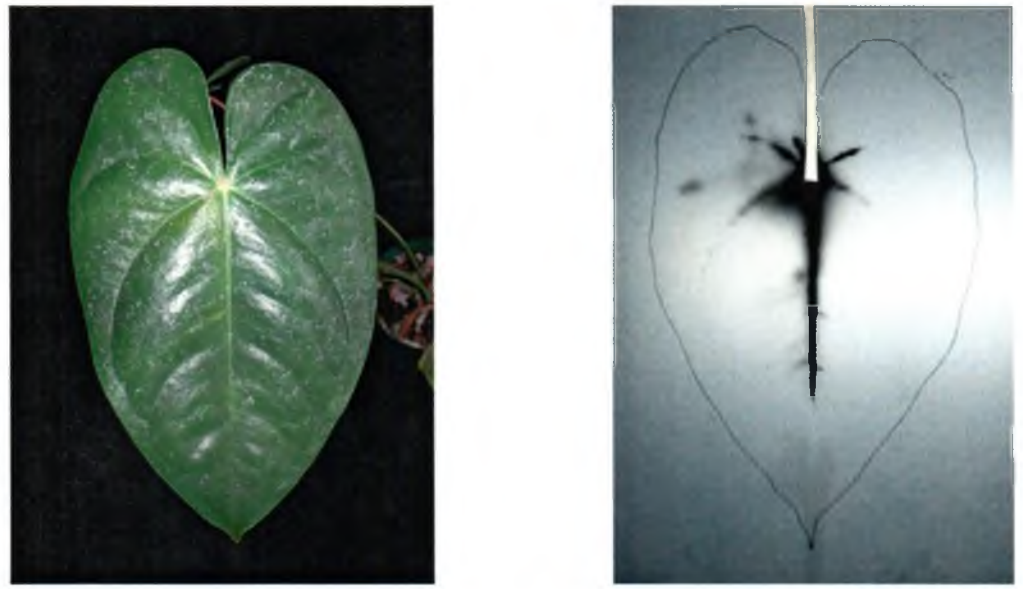
3.2.3 Leaf inoculation

Inoculum was prepared by growing *Xcd* Lux on yeast dextrose calcium carbonate (YDC) medium (Appendix B) solidified with agar for 2 days at 28 °C. Bacterial cells were resuspended in sterile distilled water, and the cell concentration of the suspension adjusted to approximately 10^8 colony forming units (CFU)/ml with a spectrophotometer (Absorbance at 600 nm = 0.1) for Experiment I and diluted to 10^5 CFU/ml for Experiment II. Individual plants were placed in plastic bags and spray-inoculated on to every leaf until run-off occurred. Control plants were sprayed with sterile distilled water. Bags were sealed and the plants were incubated overnight at 23 °C room temperature. The next day, the plants were removed from the plastic bags and placed into a greenhouse

at the University of Hawaii's Pope Laboratory. Plants were shaded with two layers of saran sheet (70% shading each) and watered by hand every other day. Average minimal and maximum temperatures were 20.1 to 27.9 °C and 23.9 to 31.6 °C during Experiments I and II, respectively.

3.2.4 Monitoring and quantifying leaf infection

A disease severity index was used based on the severity of leaf infection (percent leaf area infected), assessed by bioluminescence of the pathogen in the two youngest fully open leaves (leaf 0 and leaf 1) of each plant. Leaves emitting light produced by the bacteria were exposed to X-ray film (Fuji "New RX" medical X-ray film) by attaching the film to the underside of the leaf with plastic paper clips. Each film was covered with construction paper to protect the film from neighboring leaves emitting bioluminescence. The entire process was completed in the dark and the film was exposed for 7-8 hours. After the film was developed the shape of the leaf was traced onto the corresponding film. Infection sites on the leaf appeared as black areas on the otherwise clear film (Fig. 3-3). This method would detect disease progression in the leaf both as primary infection site (Fig. 3-3b) and as well as secondary site following systemic infection elsewhere in the plant (Fig. 3-3a). The area of infection on the film was then compared visually with a set of standard diagrams (Fig. 3-4), provided by A. Alvarez (Fukui et al., 1996), representing percentage leaf area infected (2.5, 5, 10, 15, 20, 25, 35, 45, 55, 65, 75, and 85%). Leaves were monitored for infection beginning at the fourth and sixth week post-inoculation for Experiment I and II, respectively. Assessments of disease severity were performed at least three times in each experiment. In Experiment II, disease incidence



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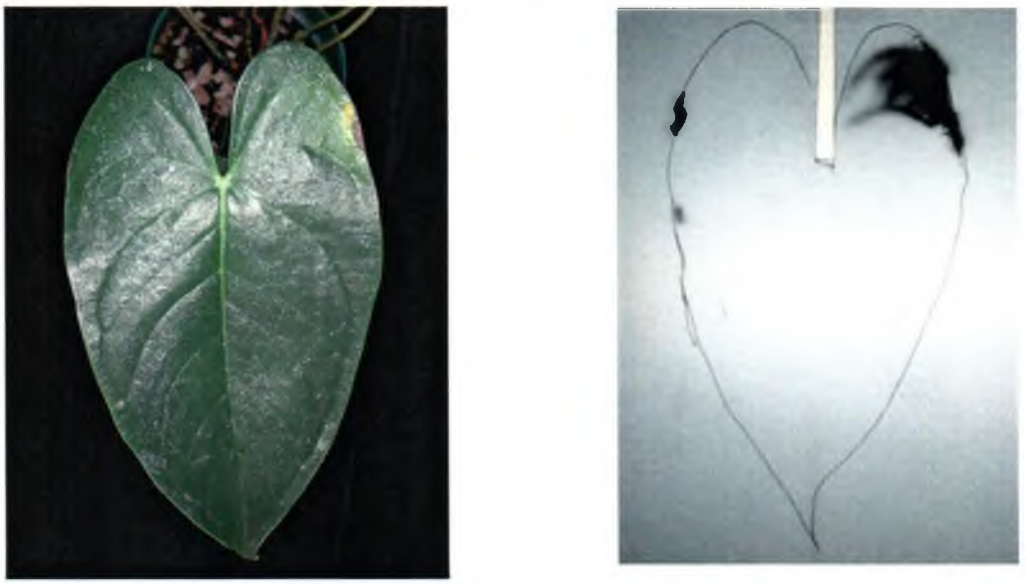


B

Fig 3-3. Anthurium leaves infected with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachiae* and corresponding X-ray film detecting bioluminescence of invading bacteria. (A) 'Tropic Flame' (UH 712) with pronounced systemic infection although foliar symptoms are absent. (B) 'Rudolph' (UH 965) with marginal infection and early colonization of vascular tissue.

Fig. 3-4. Diagram representing 2.5, 5, 10, 15, 20, 25, 35, 45, 55, 65, 75, and 85% leaf area infected with bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachiae* (Fukui et al., 1996).

was noted at each monitoring date as the number of replicated plants per line showing symptoms on the two leaves examined for bioluminescence. At some time points the necrosis due to blight is evident but the X-rays are negative for bioluminescence. At twelve weeks post-inoculation additional data was recorded with the assistance of A. Alvarez on blight symptoms assessed visually. For each plant the percentage of leaves showing visual symptoms and the total number of infection sites (based on three to four leaves per plant) were recorded. The data for the visual assessment of disease is presented in Appendix E.

3.2.5 Statistical analysis

Statistical analysis for disease severity was performed using percent infection data from leaf 0 and leaf 1. In Experiment I mean values of six replicates of each anthurium line were subjected to analysis of variance using the general linear model (GLM) procedure of SAS (SAS Institute, Cary, N.C.). In Experiment II mean values of five to ten replicates of each anthurium line were subjected to analysis of variance using the non-parametric general linear model (RANK) of SAS. The RANK procedure assigned rankings to the percentage data and the rankings were subjected to analysis of variance. Mean rankings in both experiments were separated by the Student-Newman-Keuls (SNK) test.

3.3 RESULTS

3.3.1 Experiment I

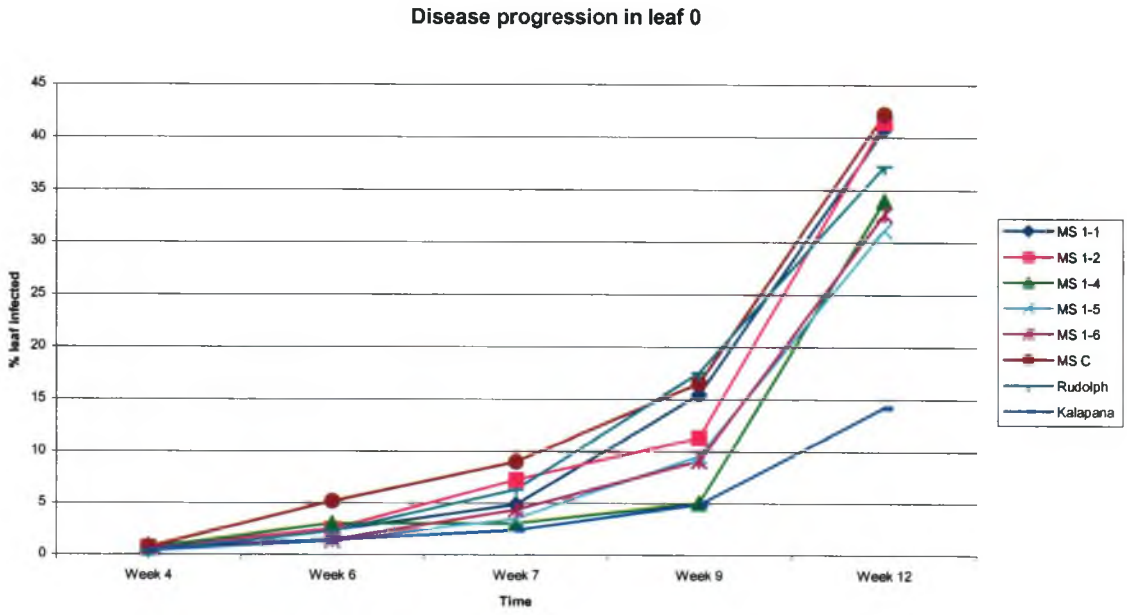
Comparison of disease severity among 'Marian Seefurth' control, transgenic lines, 'Rudolph' and 'Kalapana'.

Leaf infection was monitored at 4, 6, 7, 9, and 12 weeks post-inoculation for transgenic MS lines, MS control, 'Kalapana', and 'Rudolph'. At four weeks post-inoculation all lines displayed foliar symptoms and by week 12 all lines including the resistant check 'Kalapana' were systemically infected. However, 'Kalapana' consistently had the lowest disease severity in leaf 0 and leaf 1 at all monitoring dates (Fig. 3-5).

According to the analysis of variance, the effects of plant line on disease severity (assessment of leaf 0 and leaf 1) at all dates were not significantly different at $P \leq 0.05$ (Table 3-2). However, when symptoms in leaf 1 were evaluated over time, among transgenic lines disease seemed to progress slower in MS 1-2 and MS 1-4 from four to nine weeks post-inoculation (Fig. 3-5b). These two lines were selected for a second bacterial challenge in Experiment II.

Comparison of disease severity among UH 712 transgenic lines and control

Leaf infection was monitored at 6, 8 and 12 weeks post-inoculation. At six weeks post-inoculation no disease was detected by bioluminescence in any of the anthurium lines. At eight and twelve weeks symptoms were detectable although disease severity was not significantly different ($P \leq 0.05$) among the lines (Table 3-3). When disease progression in leaf 0 and leaf 1 was plotted three transgenic lines (712 1-1, 712 1-8, and 712 1-9) seemed to show delayed symptom development (Fig 3-6). UH 712 1-1 and 1-9 were selected for a second challenge along with 712 1-16, a line showing the greatest



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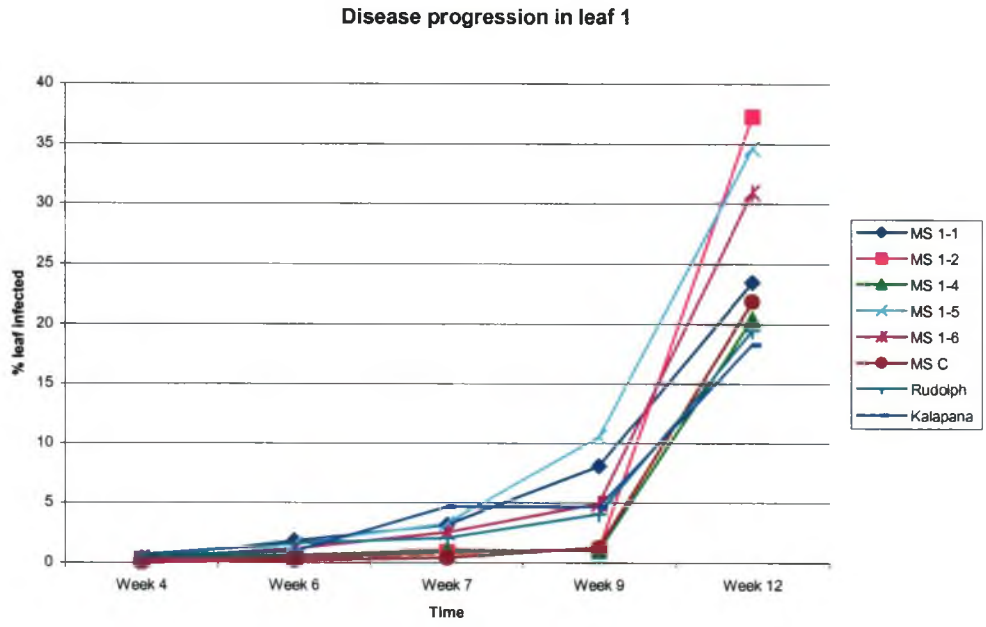


B

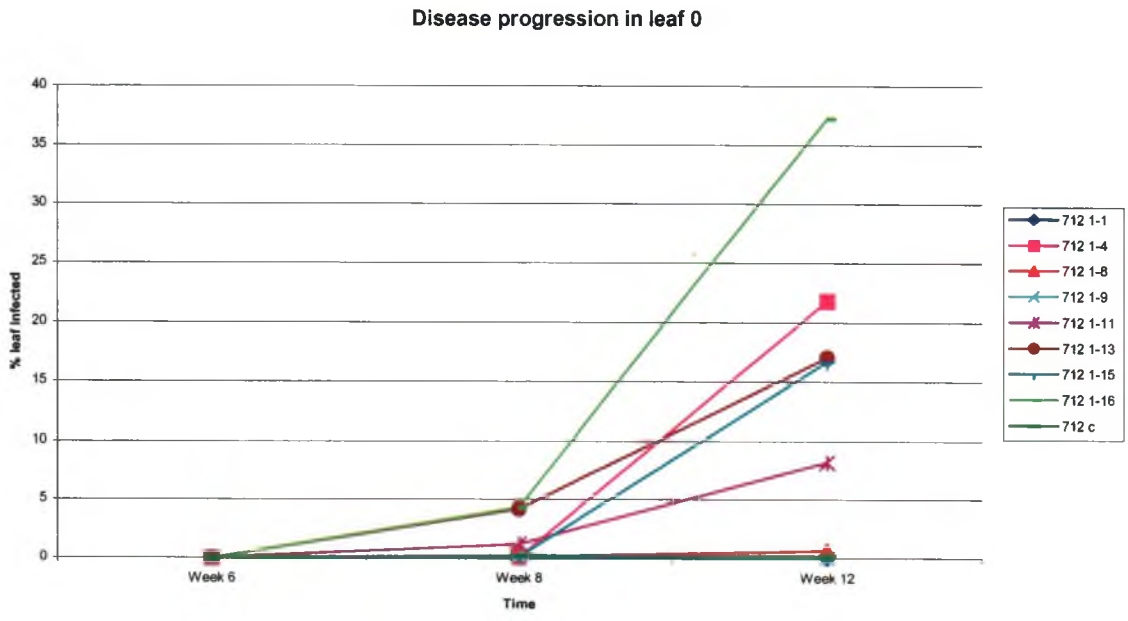
Fig. 3-5. Disease progression in transgenic and control lines of 'Marian Seefurth', 'Kalapana' and 'Rudolph' as determined by percent leaf area infected with *Xcd* Lux. (A) disease progression in leaf 0, (B) disease progression in leaf 1.

Table 3-2. Mean disease severity as percentage leaf infected of leaf 0 and leaf 1 of 'Marian Seefurth' transgenic and control lines, 'Rudolph', and 'Kalapana' inoculated with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachia* at 10^8 CFU/ml. Means of all data were not significantly different by Student Newman Keuls test at $P \leq 0.05$.

	week 4		week 6		week 7		week 9		week 12	
Plant line	leaf 0	leaf 1	leaf 0	leaf 1	leaf 0	leaf 1	leaf 0	leaf 1	leaf 0	leaf 1
MS 1-1	0.8	0.3	2.3	1.8	4.9	3.2	15.5	8.1	40.8	23.4
MS 1-2	0.7	0.3	2.5	0.3	7.3	0.9	11.3	1.1	41.4	37.3
MS 1-4	0.6	0.4	3.0	0.6	3.0	1.0	5.0	1.0	33.9	20.4
MS 1-5	0.5	0.5	1.3	1.6	3.5	3.3	9.6	10.5	31.1	34.6
MS 1-6	0.7	0.3	1.4	1.2	4.3	2.5	9.1	4.9	32.6	31.0
MS C	0.7	0.0	5.2	0.2	9.0	0.4	16.5	1.3	42.2	21.8
Rudolph	0.4	0.7	2.3	1.6	6.3	2.0	17.5	4.0	37.3	19.4
Kalapana	0.3	0.5	1.4	0.9	2.4	4.7	4.9	1.0	14.2	18.3

Table 3-3. Mean disease severity as percentage leaf infected of leaf 0 and leaf 1 of control and transgenic lines of 'Tropic Flame' (UH712) inoculated with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachia*. Means of all data were not significantly different by Student Newman Kuels test at $P \leq 0.05$

	week8		week 12	
Plant line	leaf 0	leaf 1	leaf 0	leaf 1
712 1-1	0.0	0.0	0.0	0.4
712 1-4	0.1	0.4	21.8	24.5
712 1-8	0.1	0.1	0.6	0.0
712 1-9	0.2	0.3	0.0	0.3
712 1-11	1.2	0.2	8.2	2.9
712 1-13	4.2	0.0	17.0	16.7
712 1-15	0.1	0.1	16.7	16.7
712 1-16	4.3	0.4	37.3	33.3
712 c	0.2	3.1	0.2	0.2



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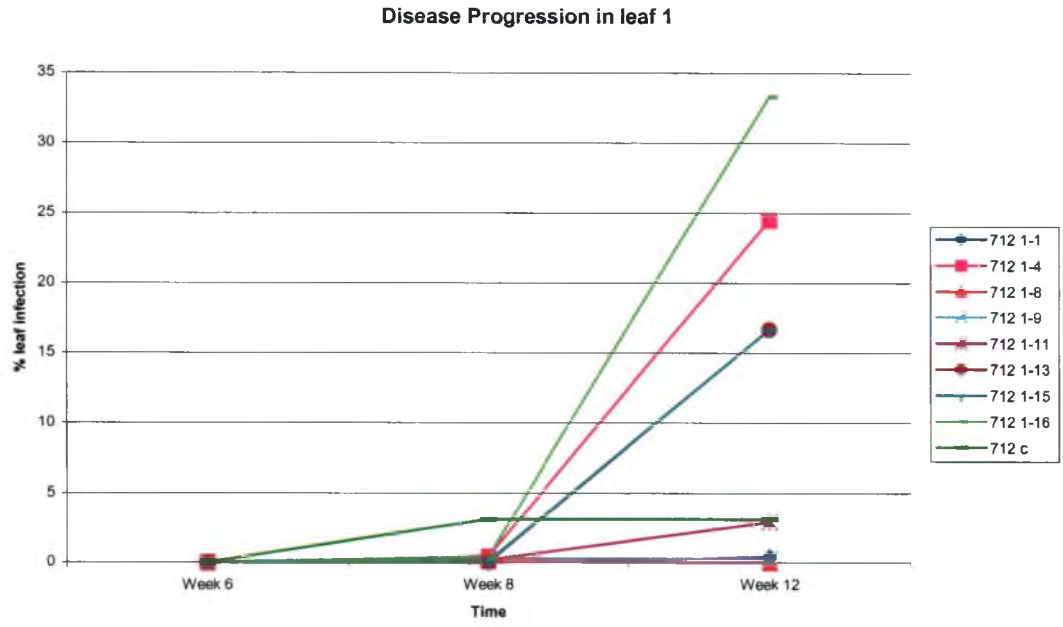


B

Fig. 3-6. Disease progression in transgenic and control lines of 'Tropic Flame' (UH 712) as determined by percent leaf area infected with *Xcd* Lux. (A) disease progression in leaf 0, (B) disease progression in leaf 1.

symptoms at week 12 (Table 3-3 and Fig. 3-6). Clonal plants of UH 712 1-8 were not available for a second challenge.

3.3.2 Experiment II

'Marian Seefurth' lines MS 1-2 and MS 1-4 were selected among the five transgenic lines to be re-evaluated based on the slower rate of disease progression and, although not significant, the lower disease severity observed in leaf 1. UH 712 lines, 1-1 and 1-9 were also selected based on their apparent slower rate of disease progression and lesser severity of infection. UH 712 1-16 was included to confirm its suspected higher susceptibility to *Xcd* Lux compared to the other UH 712 transgenic lines and control.

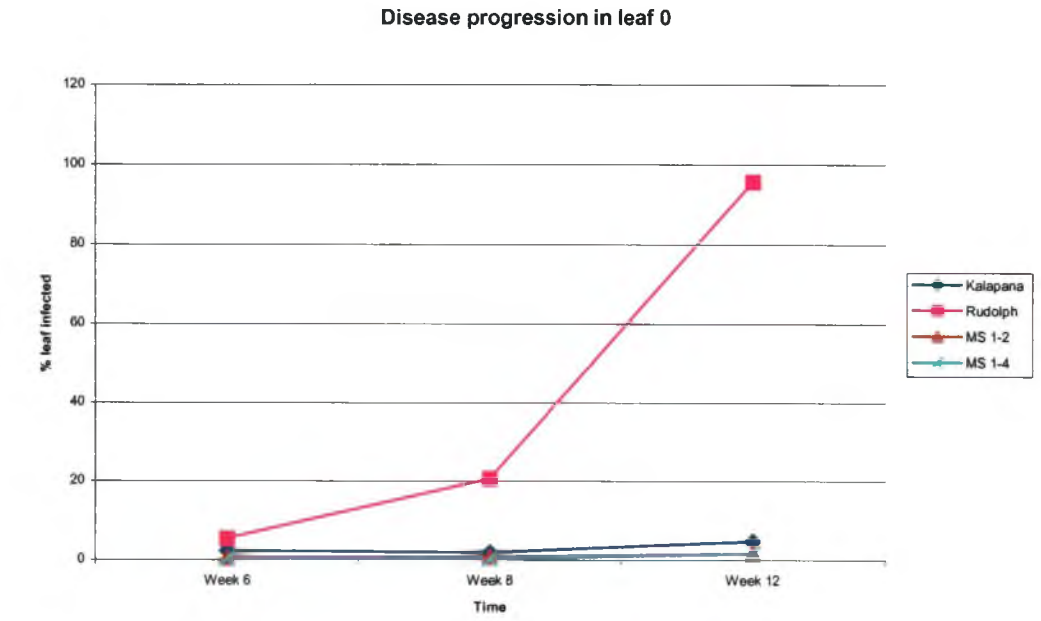
Comparison of disease severity among 'Marian Seefurth' control, transgenic lines, 'Rudolph' and 'Kalapana'.

At six weeks post-inoculation, rankings of disease severity index were not significantly different at $P \leq 0.05$ among anthurium lines. Severity of infection ranged from 0.3 to 5.0 % in leaf 0 and 0 to 11.8 % in leaf 1. Although severity of disease was similar in all lines, the disease incidence ranged from 0 to 43% (Table 3-4). At eight weeks, infection advanced markedly in 'Rudolph' (21%, 37% leaf 0 and leaf 1 respectively; Fig. 3-7), and disease severity in leaf 0 was significantly higher in 'Rudolph' than the other lines ($Pr>F = 0.0006$; Table 3-5a). Disease incidence in 'Rudolph' increased to 60% and remained the same in the other lines as six weeks post-inoculation (Table 3-4).

Trends continued into 12 weeks post-inoculation with leaf 0 means of 'Kalapana' separating from MS transgenic lines. At 12 weeks, the last monitoring date, leaf 0

Table 3-4. Disease incidence as number of plants exhibiting blight symptoms among transgenic 'Marian Seefurth' lines, 'Rudolph', and 'Kalapana' (based on 5 to 10 plant replicates).

	week 6		week 8		week 12	
Plant line	leaf 0	leaf 1	leaf 0	leaf 1	leaf 0	leaf 1
MS 1-2	2/10 (20.0%)	0/10 (0%)	2/10 (20.0%)	0/10 (0%)	3/10 (30.0%)	1/10 (10.0%)
MS 1-4	1/8 (12.5%)	2/8 (25.0%)	1/8 (12.5%)	2/8 (25.0%)	1/8 (12.5%)	2/8 (25.0%)
Kalapana	2/5 (40.0%)	2/5 (40.0%)	3/5 (60.0%)	3/5 (60.0%)	5/5 (100%)	4/5 (75.0%)
Rudolph	3/7 (42.8%)	3/7 (42.8%)	3/7 (42.8%)	3/7 (42.8%)	7/7 (100%)	7/7 (100%)



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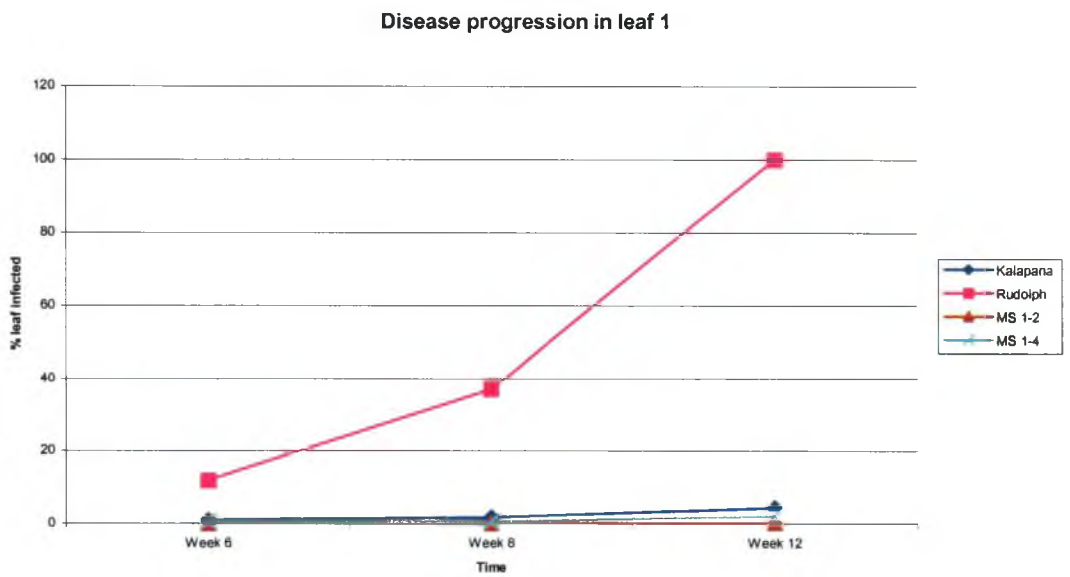

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Fig. 3-7. Disease progression in transgenic lines of 'Marian Seefurth', 'Kalapana' and 'Rudolph' as determined by percent leaf area infected with *Xcd* Lux. (A) disease progression in leaf 0, (B) disease progression in leaf 1.

showed 'Rudolph' having the greatest disease severity followed by 'Kalapana' then the two transgenic 'Marian Seefurth' lines (Table 3-5a). No significant difference in disease resistance was determined between MS 1-2 and MS 1-4. In addition, 100% of 'Rudolph' and 'Kalapana' replicate plants were infected in leaf 0 compared to 30% and 12.5% of MS 1-2 and MS 1-4 respectively (Table 3-4). Based on the disease severity index of leaf 0, transgenic MS plants displayed higher resistance to *Xcd* Lux than the blight susceptible 'Rudolph' and the blight resistant cultivar 'Kalapana'.

Comparison of disease severity among UH 712 transgenic lines and control.

At six weeks post-inoculation, disease severity (% leaf infection) in leaf 0 of 712 1-16 was significantly higher ($Pr>F = 0.0028$) than the other lines. UH 712 1-16 leaf 0 had a disease severity index of 4% compared to 0-1.7% in the other lines (Table 3-6a) and 5.6% infection in leaf 1 compared to 0-1.5% of the other 712 lines (Table 3-6b). Sixty-seven percent of 712 1-16 plants displayed disease symptoms in leaf 0 compared to 0, 10, and 20% of 712 1-1, control and 712 1-9, respectively (Table 3-7). Fifty-six percent of replicated 712 1-16 plants shown infection in leaf 1 compared to 0, 10 and 40% of 712 1-1, control, and 712 1-9 respectively (Table 3-7). Trends toward higher disease severity in 712 1-16 continued through the two subsequent monitoring dates (Fig. 3-8a,b).

At eight weeks post-inoculation, 712 1-16 mean severity index increased to 15% (Fig. 3-8a,b), which was significantly higher ($Pr>F = 0.0004$, leaf 0 and $Pr>F = 0.0147$, leaf 1) than the severity index of the other 712 lines (Table 3-6). Mean disease severity index of 712 control, 712 1-1 and 712 1-9 ranged from 0 to 5% for leaf 0 and leaf 1 and

Table 3-5. Mean disease severity as a percentage leaf infected and statistical ranking of control and transgenic lines of anthuriums inoculated with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachia* at 10^5 CFU/ml. (A) leaf 0 and (B) leaf 1. Means of 10 replicates with same letter in a row were not significantly different by Student Newman Keuls test at $P \leq 0.05$.

	week 6		week 8		week 12	
Plant line	% infection	ranking	% infection	ranking	% infection	ranking
MS 1-2	0.5	14.3 a	0.8	12.7 b	1.6	10.7 c
MS 1-4	0.3	13.3 a	0.3	10.5 b	1.5	9.3 c
Kalapana	2.3	18.1 a	1.9	16.3 b	4.7	18.0 b
Rudolph	5.4	17.9 a	20.7	24.7 a	95.7	26.0 a

A

	week 6		week 8		week 12	
Plant line	% infection	ranking	% infection	ranking	% infection	ranking
MS 1-2	0.0	14.9 a	0.0	9.5 c	0.1	9.4 c
MS 1-4	0.5	11.5 a	0.4	12.4 bc	1.9	11.3 bc
Kalapana	0.9	18.1 a	1.6	18.0 ab	4.2	15.7 b
Rudolph	11.8	18.4 a	37.0	22.3 a	100.0	24.5 a

B

Table 3-6. Mean disease severity as percentage leaf infected and statistical ranking of control and transgenic lines of 'Tropic Flame' (UH 712) inoculated with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachia*. (A) leaf 0 and (B) leaf 1. Means of 10 replicates with same letter in a row were not significantly different by Student Newman Keuls test at $P \leq 0.05$.

	week 6		week 8		week 12	
Plant line	% infection	ranking	% infection	ranking	% infection	ranking
712 1-1	0.0	15.5 b	0.0	15.0 b	0.0	13.5 b
712 1-9	1.7	19.5 b	5.4	18.8 b	12.8	18.2 b
712 1-16	4.0	28.3 a	15.0	29.3 a	44.7	29.2 a
712 c	0.9	17.6 b	1.5	16.9 b	4.9	17.9 b

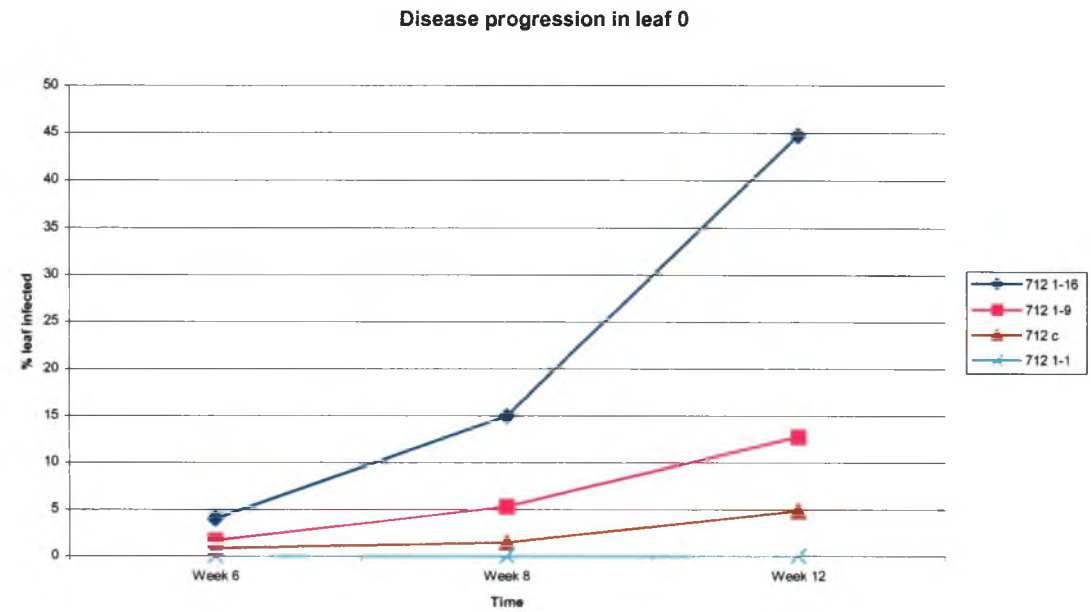
A

	week 6		week 8		week 12	
Plant line	% infection	ranking	% infection	ranking	% infection	ranking
712 1-1	0.0	15.0 b	0.0	14.5 b	0.2	16.0 b
712 1-9	1.3	21.8 ab	3.0	16.7 b	4.8	16.7 b
712 1-16	5.6	26.6 a	14.6	26.1 a	40.8	28.8 a
712 c	1.5	17.3 b	4.1	20.9 b	5.5	19.5 b

B

Table 3-7. Disease incidence as number of plants exhibiting blight symptoms among transgenic 'Tropic Flame' (UH 712) lines and control (based on 9 to 10 plant replicates).

	week 6		week 8		week 12	
Plant line	leaf 0	leaf 1	leaf 0	leaf 1	leaf 0	leaf 1
712 1-1	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10.0%)
712 1-9	2/10 (20.0%)	1/10 (10.0%)	2/10 (20.0%)	1/10 (10.0%)	2/10 (20.0%)	1/10 (10.0%)
712 1-16	6/9 (66.7%)	5/9 (55.5%)	6/9 (66.7%)	5/9 (55.5%)	7/9 (77.8%)	6/9 (66.7%)
712 c	1/10 (10.0%)	4/10 (40.0%)	1/10 (10.0%)	4/10 (40.0%)	2/10 (20.0%)	4/10 (40.0%)



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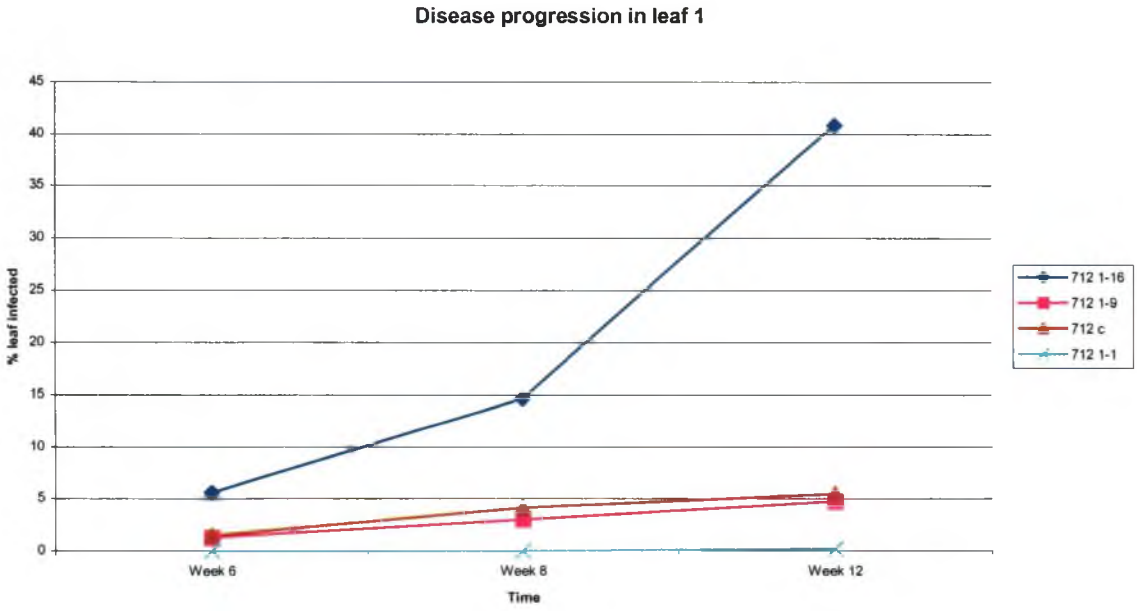

B

Fig. 3-8. Disease progression in transgenic and control lines of 'Tropic Flame'(UH 712) as determined by percent leaf area infected with *Xcd* Lux. (A) disease progression in leaf 0, (B) disease progression in leaf 1.

were not statistically different. Disease incidence in all lines did not change from six weeks post-inoculation (Table 3-7).

Disease severity index increased to about 45% for 712 1-16 at 12 weeks post-inoculation (Fig. 3-8a,b), which was again significantly higher ($Pr>F = 0.0005$, leaf 0 and $Pr>F = 0.0038$, leaf 1) than the other 712 lines (Table 3-6). The remaining plant disease severity indices ranged from 0 to 13%. Disease incidence in leaf 0 rose from 67% at week 8 to 78% and week 12 in 712 1-16 and 10 to 20% in 712 1 control; it remained the same for 712 1-1 and 712 1-9 (Table 3-7). Percentage of leaf 1 infected plants increased about 10% for 712 1-1 and 712 1-16. Disease incidence in leaf 1 of 712 1-9 and 712 control did not change from 8 weeks to 12 weeks post-inoculation (Table 3-7). UH 712 1-1 consistently had the lowest amount of replicated plants diseased with only 10% of the replicates diseased at the termination of the experiment.

3.4 DISCUSSION AND CONCLUSION

Upon flowering, the transgenic MS lines appeared to be another equally susceptible pink cultivar, 'Paradise Pink' and not 'Marian Seefurth' as originally labeled. However, it should be stressed that the results section refers to these plants as 'Marian Seefurth' to match the MS line number designation. Disease resistance of two anthurium cultivars transgenic for Shiva-1 was evaluated by inoculation with a bioluminescent strain of the blight bacteria *Xanthomonas campestris* pv. *dieffenbachiae*. Two transgenic lines of 'Paradise Pink' (originally labeled 'Marian Seefurth', MS 1-2 and MS 1-4) displayed enhanced resistance to bacteria blight over blight susceptible 'Rudolph' and

even the blight resistant 'Kalapana'. Neither 'Marian Seefurth' nor 'Paradise Pink' control plants were available for comparison at the time of the second experiment. When evaluating disease resistance in transgenic lines of 'Tropic Flame', one line, 712 1-16 unexpectedly was the most susceptible of all lines including the control (Fig. 3-9). Other 'Tropic Flame' transgenic lines showed no improved resistance when compared to the control at the mean percent leaf infection level.

Similar to the high susceptibility of UH 712 1-16, Jaynes et al. (1993) also observed elevated disease symptoms in tobacco plants transgenic for Shiva-1 when inoculated with *Pseudomonas solanacearum* (Jaynes et al., 1993). They attributed this to a cell proliferation effect of peptides at low concentrations. In vitro, Peptidyl MIM 2L2, another synthetic cecropin analog, increased cell titers of *Acholeplasma laidlawii* 20 to 30% (Borth et al., 2001). Our Shiva-1 in vitro assay also showed proliferation of certain bacteria species in the presence of low Shiva-1 concentrations. Tests are ongoing to determine if UH 712-16 has lower expression of Shiva-1 in relation to the other transgenic lines, which is hypothesized to be the cause of its increased susceptibility to *Xcd* Lux.

The molecular analysis completed prior to disease challenging gave every indication that plants harboring and/or expressing the gene of interest (Shiva-1) were included in the inoculation trials. Replicates of each transgenic line gives us confidence that the differences in disease response are significant. Nevertheless, a range of responses was detected within almost every line. This indicates the complex nature of the anthurium-*Xcd* interaction and emphasizes the value of quantifying disease progression using bioluminescence. To better understand the results of both challenge

A

B

Fig. 3-9. 'Tropic Flame' (UH 712) leaves infected with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachiae*. (A) Transgenic line UH 712 1-1, and (B) Transgenic line UH 712 1-9.

C

D

Fig. 3-9 (cont.). 'Tropic Flame' (UH 712) infected with a bioluminescent strain of *Xanthomonas campestris* pv. *dieffenbachiae*. (C) Transgenic line UH 712 1-16, and (D) UH 712 control non-transgenic.

experiments at the two inoculum densities, it would be useful to understand if transgenic plants were not producing Shiva-1 or producing the peptide at various levels. Experiments to determine the expression of the Shiva-1 at the RNA level by Northern analysis are being conducted to confirm these results.

Methods of analyzing resistance in anthuriums can also be improved. Monitoring two leaves per plant can be misleading and at times is not an accurate representation of disease severity of the entire plant. In many cases, leaves not subjected to X-ray analysis displayed blight symptoms. This was evident when disease incidence (percentage of replicated plants showing symptoms) was higher when monitored visually versus the two leaf bioluminescent method of analysis. Although labor intensive, X-ray analysis of all leaves will give a better idea of a plant's susceptibility or tolerance to bacterial blight.

In conclusion, transgenic 'Paradise Pink' displayed enhanced resistance to bacterial blight over 'Kalapana', a naturally tolerant cultivar, and over susceptible check 'Rudolph'. On the other hand, genetic engineering of 'Tropic Flame' (UH 712) has the potential to render the cultivar more susceptible to the disease. Based on these results, genetic engineering with Shiva-1 may enhance resistance to bacterial blight for only certain cultivars of anthurium.

3.5 LITERATURE CITED

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CHAPTER 4

EFFECT OF TRANSGENIC ANTHURIUMS PRODUCING THE SHIVA-1 LYTIC PEPTIDE ON POPULATIONS OF BENEFICIAL PLANT-ASSOCIATED BACTERIA

4.1 INTRODUCTION

Over the recent years much effort has focused on genetically modifying plants for enhanced disease resistance with lytic peptides (Hancock and Lehrer, 1998; Mourgues et al., 1998; Rao, 1995). However, studies on the consequences of these genetically engineered plants on non-target organisms in the natural microflora are rare. Many of these non-target organisms are bacteria that live in close association with plants and can deeply influence plant health by suppressing disease, promoting growth, and enhancing nutrient uptake (Smith et al., 1999).

Lottmann et al. (1999) evaluated the effects of transgenic T4-lysozyme-producing potatoes on populations of beneficial plant-associated bacteria in the geocaulosphere (tuber surface) and rhizosphere. They observed no alteration in counts of culturable aerobic bacteria, antagonistic bacteria and no significant differences in the ability of beneficial bacteria to produce auxins producing isolates. Lottmann also cited a study where genetically engineered potato plants producing the *Bacillus thuringiensis* endotoxin had no significant effects on the microbial species associated with the potato leaves (Donegan et al., 1996).

We have genetically engineered anthurium to express a secreted cecropin analogue, Shiva-1, in an effort to control anthurium bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae* (*Xcd*). When compared to native cecropin,

Shiva-1 has a more potent lytic activity against a wide variety of phytopathogenic bacteria (Jaynes et al., 1993). To our knowledge, no studies have been conducted to determine the effects of lytic peptides produced by transformed plants on communities of beneficial plant-associated bacteria. The first objective of this study was to determine in vitro concentrations of Shiva-1 that are lethal to beneficial bacteria and to the pathogenic *Xcd*. Since in vitro and in vivo situations are fundamentally different, in vitro results may not truly correspond to the nature of Shiva-1 in situations in vivo. Therefore, a second objective of this experiment was to determine if genetically engineered anthuriums producing Shiva-1 alter the population of four phyllosphere-residing species of beneficial plant-associated bacteria. The first experiment consisted of an in vitro bactericidal assay using commercially produced Shiva-1. The second experiment evaluated population counts of beneficial bacteria species in guttation fluid from anthuriums transgenic for Shiva-1.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial/culture

Four bacterial species (Table 4-1) were obtained from A. Alvarez, Dept of Plant and Environmental Protection Sciences, University of Hawaii. *Sphingomonas chlorophenolica* (Gut 3), *Microbacterium testaceum* (Gut 4), *Brevundimonas vesicularis* (Gut 5), and *Herbaspirillum rubrisubalbicans* (Gut 6) were originally isolated from guttation fluids of two blight susceptible anthurium hybrids, with Gut 3, Gut 4, and Gut 5 from 'Marian Seefurth' and Gut 6 from University of Hawaii hybrid UH1060 (Fukui et al., 1999a). The bacteria were cultured on YDC medium for inoculation purposes.

Table 4-1. Anthurium-derived beneficial bacteria.

Strain	Bacteria	Gram
GUT3	*Sphingomonas chlorophenolica*	-
GUT4	*Microbacterium testaceum*	+
GUT5	*Brevundimonas vesicularis*	-
GUT6	*Herbaspirillum rubrisubalbicans*	-

4.2.2 In vitro antibacterial assay

Shiva-1 lytic peptide was assayed in vitro for its antibacterial activity against the four beneficial bacteria and the anthurium blight pathogen *Xcd* using a microdilution method. Shiva-1 was purchased from BACHEM California, Torrance, CA. and serially diluted in 10 mM phosphate buffer to give concentrations ranging from 0.125 μM to 8 μM. Bacteria were grown on YDC medium solidified with agar for 2 days at 28°C. The optical density (O.D.) at 600 nm for each bacterial species adjusted to 0.1, which is equivalent to approximately 10^8 CFU/ml. The suspension was then diluted to 10^6 CFU/ml. The minimal inhibitory concentration (MIC) of Shiva-1 was determined in a 96-well tissue culture plate. Volumes of 50 μl of each concentration of peptide (8, 4, 2, 1, 0.5, 0.25, and 0.125 μM) was added to 50 μl of bacteria cells (~10^6 CFU/ml) giving the following final working concentrations of Shiva-1 at 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 μM and a bacterial concentration of 5 x 10^4 cells per well. Three replicates were made for each concentration of peptide. Fifty microliters of phosphate buffer (0 μM Shiva-1) was added to the bacteria suspension used as control. The resulting bacteria/Shiva-1 suspensions were incubated at 28°C on a rotary shaker set at 200 rpm for 16-18 hours. After incubation, the bacterial suspensions were diluted in a ten-fold dilution series (10^{-1} to 10^{-4}) and plated in 100 μl volumes (3 drops of 33.3 μl) on triphenyltetrazolium chloride (TTC) medium (Appendix C). Colonies were counted using a dissecting scope after 2 days of incubation at 28°C (Fig. 4-1). The number of bacterial colonies at each concentration of Shiva-1 was compared within each bacterial species. Mean bacterial counts were subjected to analysis of variance using the general linear model (GLM) procedure of SAS (SAS Institute, Cary, N.C.) and means were separated by the Student-

Fig. 4-1. Example of dilution plating from 10^{-1} to 10^{-4} of a suspension containing bacteria (Gut 4) and Shiva-1. Top (l-r) Shiva-1 concentrations of 4 μM, 2 μM, 1 μM, 0.5 μM, 0.25 μM, 0.125 μM, 0.0625 μM, and 0 μM.

Newman-Keuls Test. The lowest concentration of Shiva-1 that inhibited all cell growth was noted as MIC.

4.2.3 Plant material (Expt. 2)

Two cultivars of transgenic anthuriums were tested. Two transgenic lines of 'Marian Seefurth' (MS 1-1 and MS 1-5) and two lines of transgenic 'Mauna Kea' (MK 1-2 and MK 2-6), (Fig. 4-2). Each line consisted of 5 to 7 plant replicates. Transgenic anthurium lines were selected based on their nucleic acid and protein levels of Shiva-1 (data not shown). These transgenic lines were attained using *Agrobacterium* transfection of etiolated internodes (Chen and Kuehnle, 1996). All plants were approximately two years old and planted in 4" pots containing a 3:1 composted redwood bark to perlite #3 mix.

4.2.4 Plant inoculation (Expt. 2)

Inoculum was prepared by growing the bacteria for 2 days at 28°C on YDC medium solidified with agar. Cells of the four bacterial species were resuspended in sterile distilled water to a cell concentration of approximately 10^8 colony forming units per ml (CFU/ml) using a spectrophotometer (A_{600} = 0.1 O.D.) then mixed 1:1:1:1. The mixed suspension was diluted to approximately 10^6 CFU/ml and used as the inoculum. Individual plants were placed into plastic bags and spray-inoculated until runoff. The bags were sealed and plants were incubated overnight at 23 °C room temperature. The following day the plants were removed from the plastic bags and moved into a glasshouse at the University of Hawaii Pope Laboratory.

A

B

Fig. 4-2. Inflorescence of University of Hawaii anthuriums. (A) 'Marian Seefurth', (B) 'Mauna Kea'. Photographs courtesy of H. Kamemoto.

4.2.5 Isolation of Beneficial Bacteria (Expt. 2)

Beneficial bacteria reside in the hydathodes of leaves and thus are present in exuding guttation fluid. To collect guttation fluid, two youngest expanded leaves of each plant were covered with plastic bags in the late afternoon to maintain high humidity levels and to prevent transpiration. Plants were also thoroughly watered to increase root pressure overnight. The next morning droplets of guttation fluid, exuding from the hydathodes and accumulating on the leaf margins (Fig. 4-3), were collected and pooled using a micropipetter. Guttation fluid was collected 6 and 11 days post-inoculation. Cell densities of the four bacteria were identified by dilution plate counting of the guttation fluid on selective culture medium of modified TTC medium (Norman and Alvarez, 1989) containing antibiotics (Appendix D). Whenever possible, 100 µl of guttation fluid were collected and diluted with sterile 10 mM phosphate buffer in a ten-fold dilution series (0-10^{-4}). Three drops of 33.3 µl (~100 µl) of each dilution series were made on each of the selection media. Selection plates were incubated for 2 days at 28°C and bacterial colonies were counted using a dissecting scope and expressed as CFU/ml guttation fluid.

The experiment consisted of five to seven replications per plant line. Mean bacterial counts of each species compared between transgenic and control lines using the general linear model (GLM) procedure of SAS (SAS Institute, Cary, N.C.) for analysis of variance. Means were separated by the Waller-Duncan K-ratio t Test.

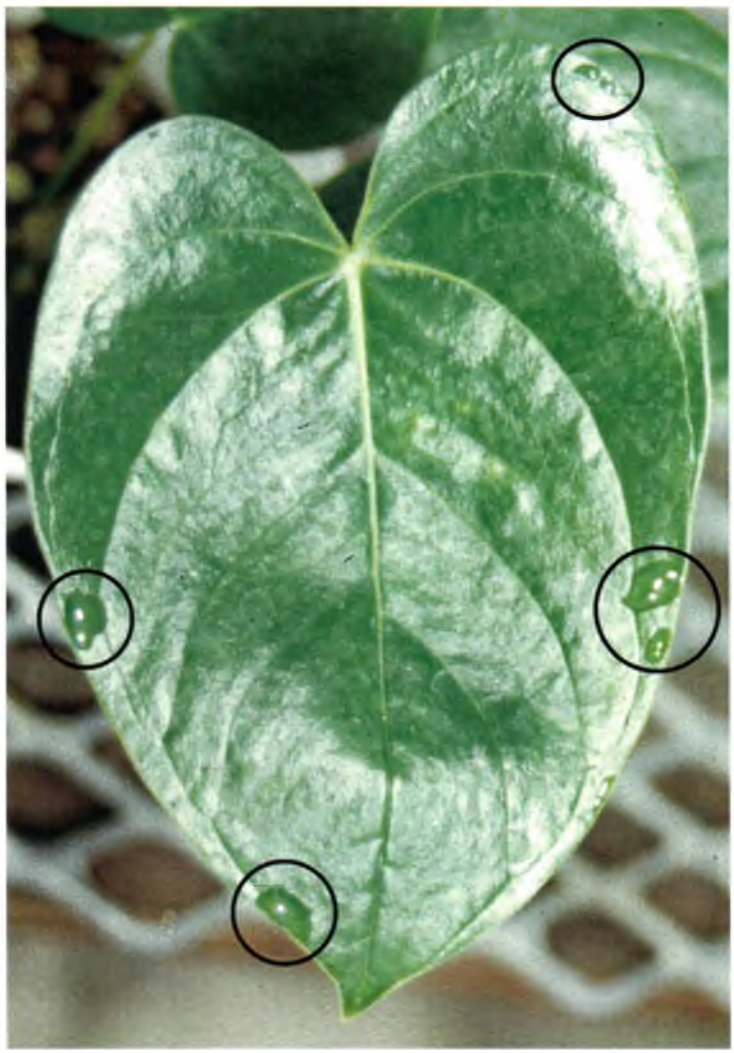

Fig. 4-3. Guttation fluid exuded from hydathodes of anthurium leaf

4.3 RESULTS

4.3.1 In vitro antibacterial assay

Inhibitory concentrations of Shiva-1 against four beneficial and one pathogenic species of bacteria was determined after 16-18 hours exposure to lytic peptide (Table 4-2).

Concentrations of Shiva-1 completely inhibiting growth of the bacteria were, 4 μM (16.96 μg/ml) Gut 3; 2 μM (8.48 μg/ml) Gut 4; 2 μM (8.48 μg/ml) Gut 5, and 2 μM (8.48 μg/ml) Xcd Lux. Some concentrations shown statistically to be as effective as the complete inhibition concentration (bacteria counts at these concentrations were not significantly different from zero growth at $P \leq 0.05$) still supported nominal colony growth (2 μM for Gut 3, 1 μM for Gut 5, and 0.5-1 μM for *Xcd* Lux). Gut 6 was the only species to have colony counts at all concentrations of Shiva-1 tested. No inhibition was observed at 4 μM Shiva-1.

Determination of the peptide concentrations required for 50% growth inhibition (IC_{50}) of bacteria gives another indication of sensitivity of different strains to Shiva-1. Gut 5 was the most sensitive ($IC_{50} \leq 0.0625$ μM) and Gut 6 was the least sensitive ($IC_{50} >$ 4 μM). When these results are considered with the IC_{100} results, the beneficial bacteria Gut 3 and Gut 6 were less sensitive to Shiva-1 then *Xcd* Lux, with Gut 4 having the same sensitivity and Gut 5 being more sensitive (Table 4-2). Results also indicate the beneficial bacteria, unlike the pathogen, have a threshold response rather than a dosage response to the different concentration of Shiva-1 used in this experiment (Figs. 4-4). An interesting effect of Shiva-1 was the increase in Gut 3 colonies at 0.5 μM Shiva-1. This proliferation effect was also evident in Gut 6 where concentrations between 0.5 and 1 μM

Table 4-2. Antibacterial activity of Shiva-1 peptide against beneficial bacteria and their sensitivity relative to pathogenic *Xanthomonas campestris pv. dieffenbachiae* (*Xcd*). IC_{50} and IC_{100} are the peptide concentrations (μM) required for 50% and 100% growth inhibition of 5×10^4 CFU and expressed as bracketed values.

Bacterium	Range for IC_{50} (μM)	Range for IC_{100} (μM)	Sensitivity
Sphingomonas chlorophenolica (Gut 3)	1 - 2	2 - 4	less than *Xcd*
Microbacterium testaceum (Gut 4)	1 - 2	1 - 2	equal to *Xcd*
Brevundimonas vesicularis (Gut 5)	≤ 0.0625	1 - 2	more than *Xcd*
Herbaspirillum rubrisubalbicans (Gut 6)	> 4	>4	less than *Xcd*
Xanthomonas campestris pv. *diffenbachiae* (*Xcd* Lux)	1 - 2	1 - 2	-

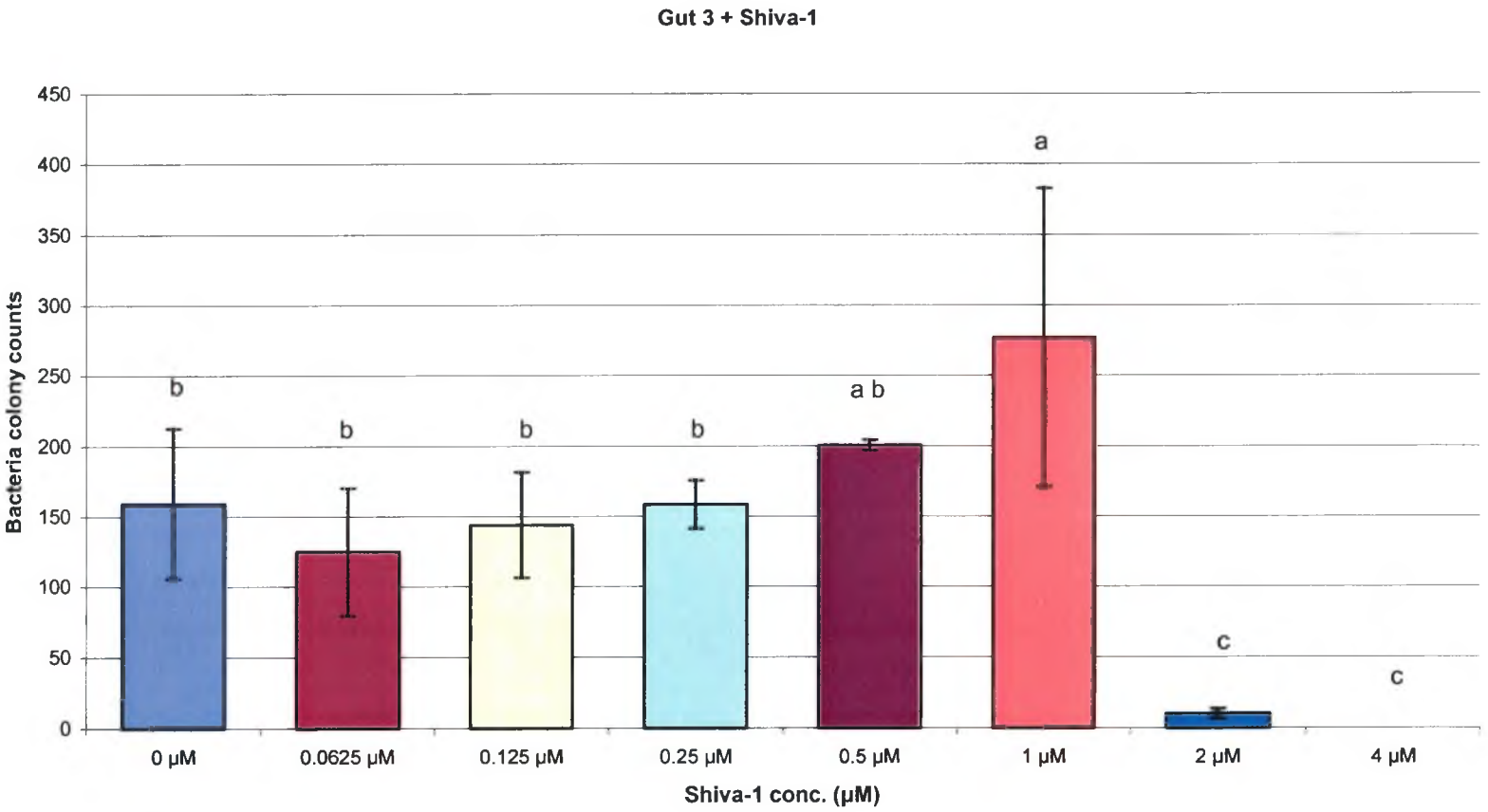

Fig. 4-4 a. Gut 3 colony counts after exposed to varying concentrations of Shiva-1 for 16-18 hrs.

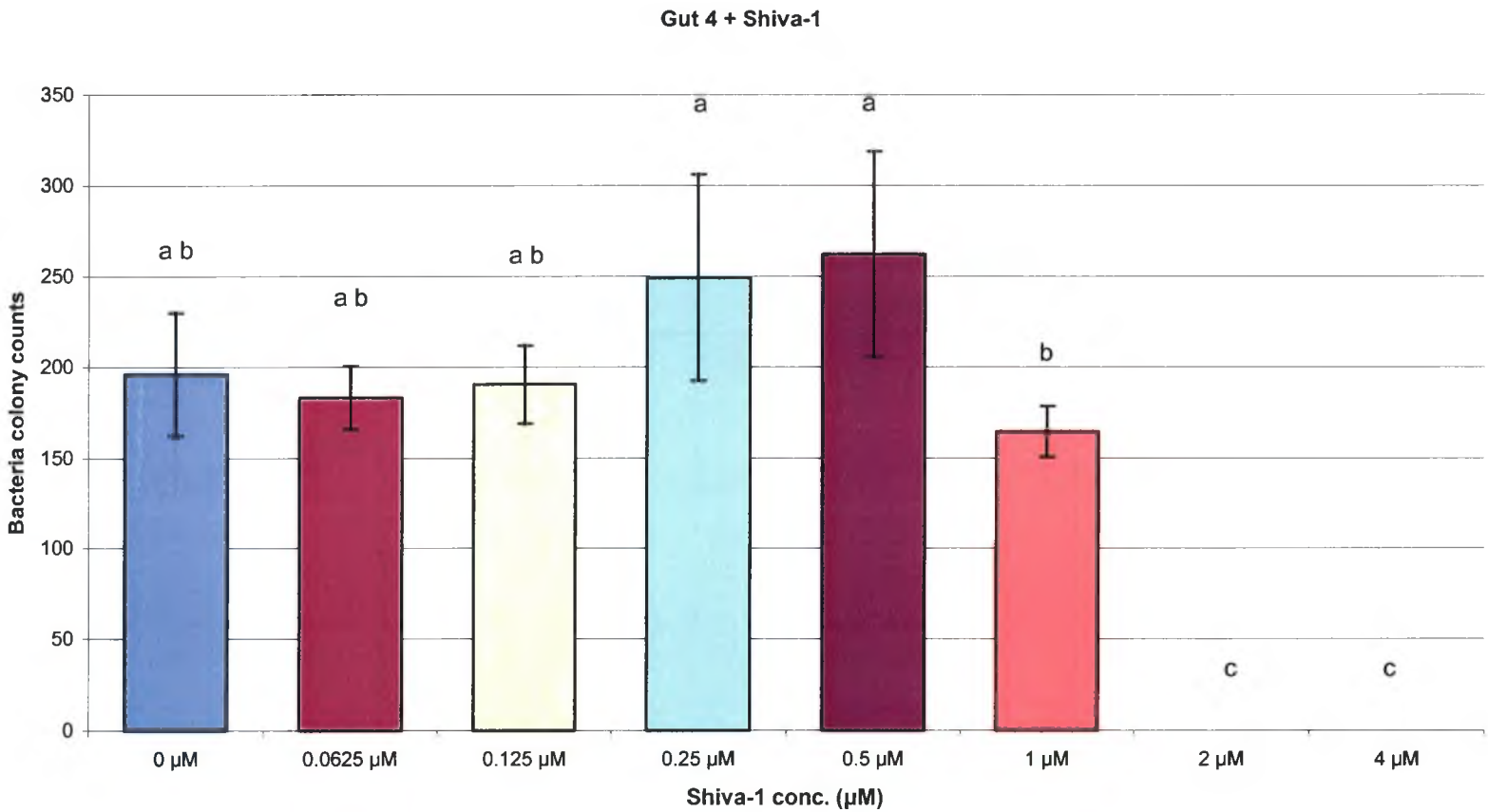

Fig. 4-4 b. Gut 4 colony counts after exposed to varying concentrations of Shiva-1 for 16-18 hrs.

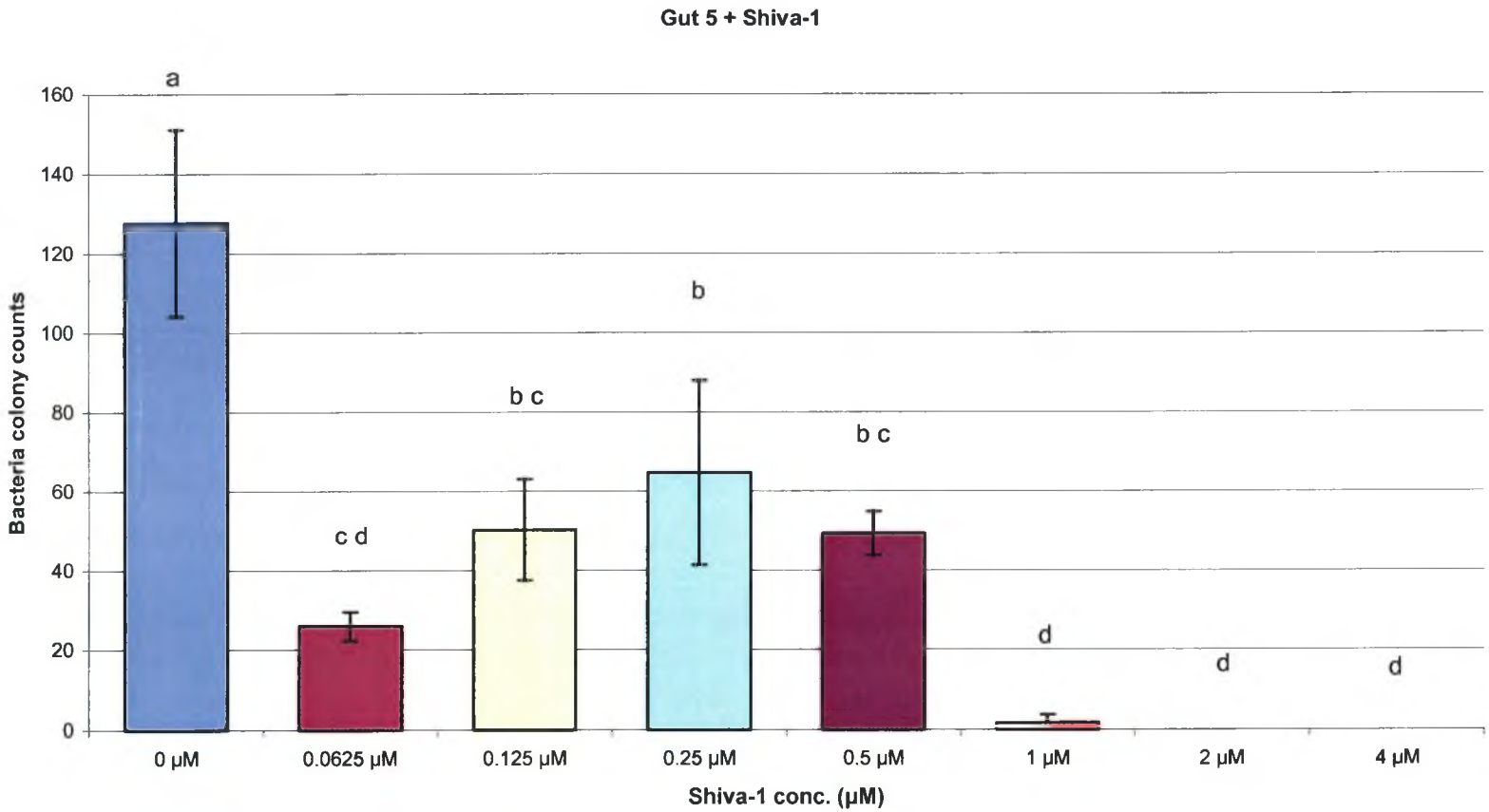

Fig. 4-4 c. Gut 5 colony counts after exposed to varying concentrations of Shiva-1 for 16-18 hrs.

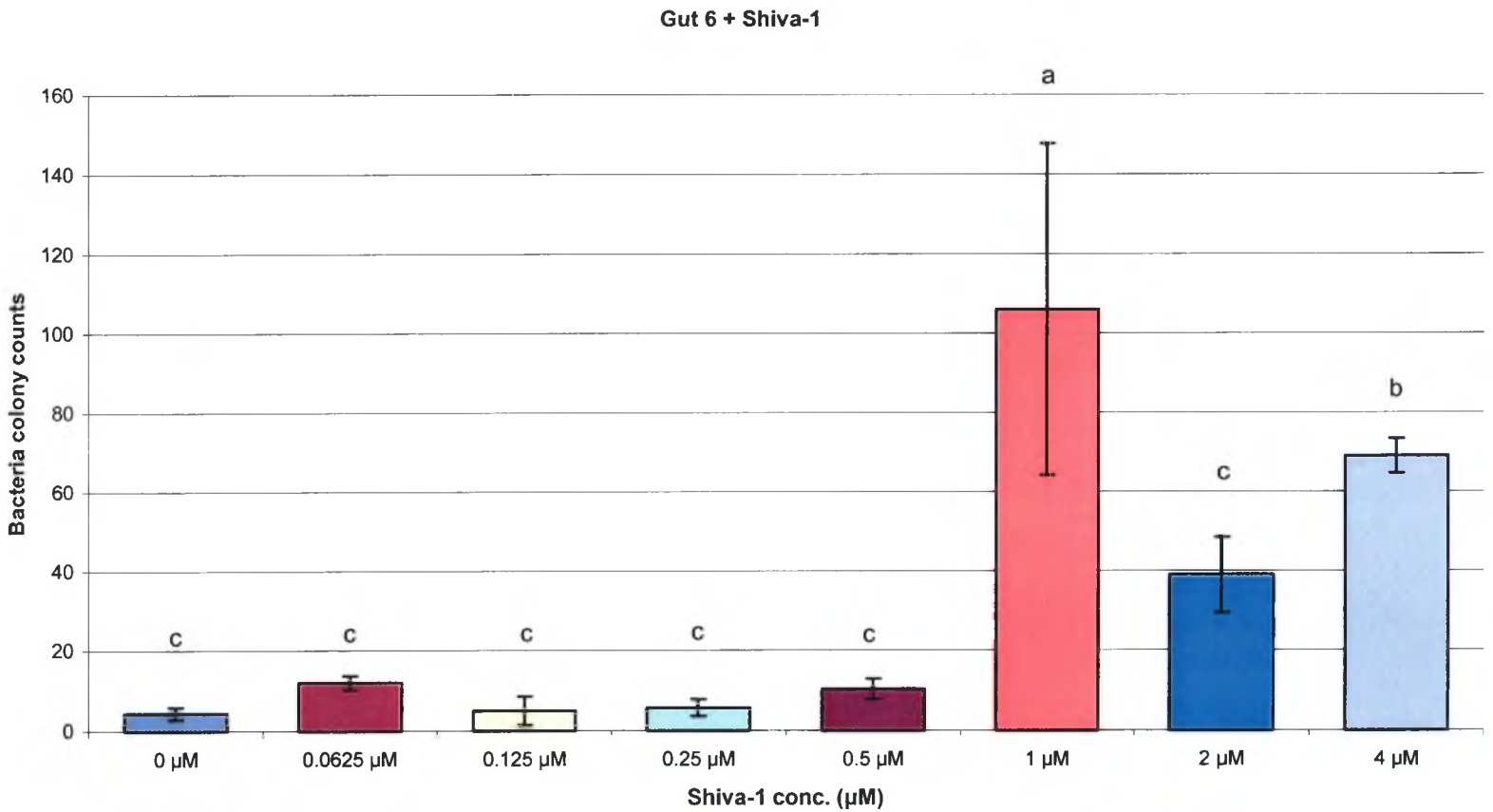

Fig. 4-4 d. Gut 6 colony counts after exposed to varying concentrations of Shiva-1 for 16-18 hrs.

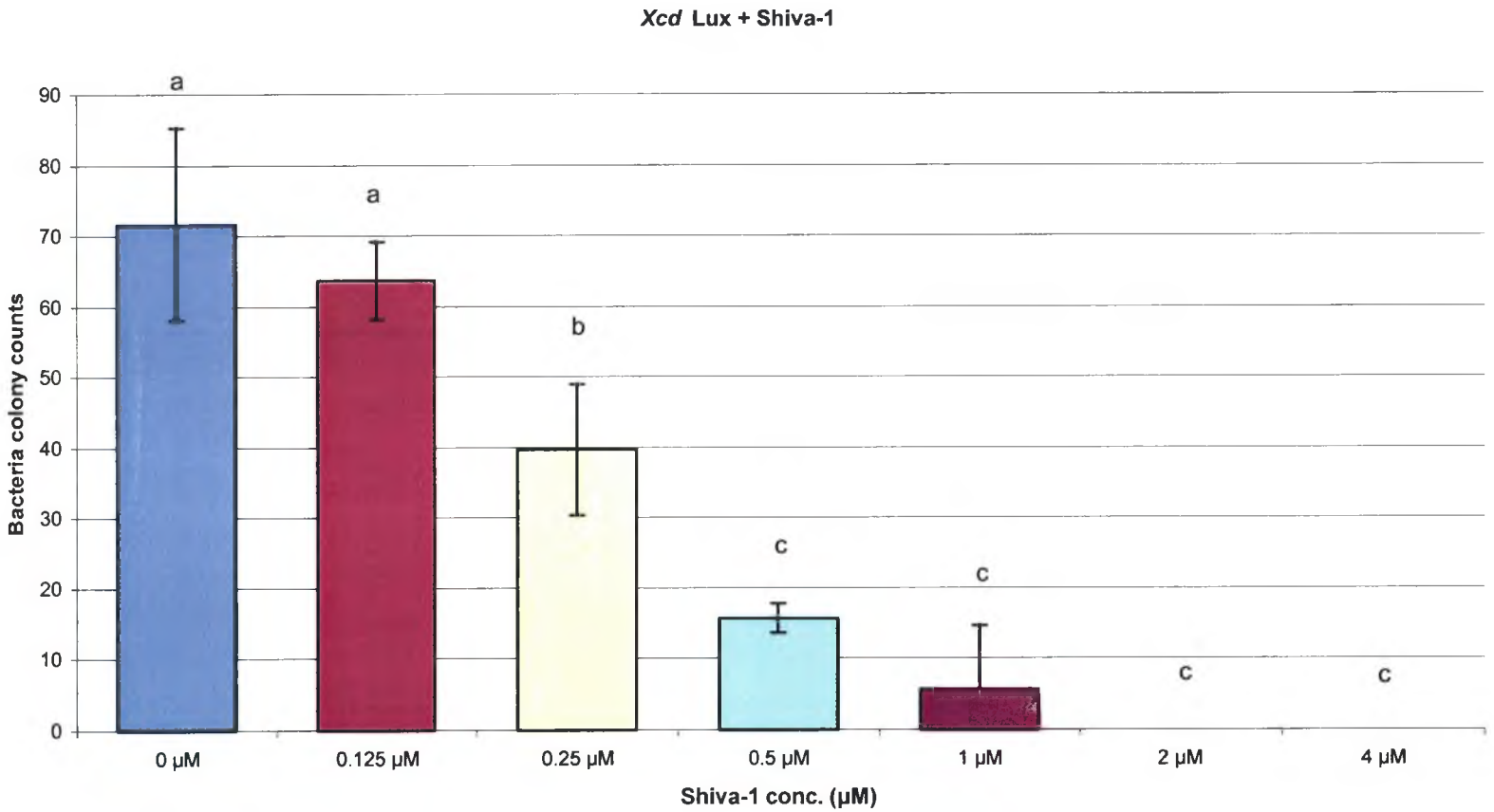

Fig. 4-4 e. *Xcd* Lux colony counts after exposed to varying concentrations of Shiva-1 for 16-18 hrs.

(and higher) and 4 μM resulted in an increase in bacteria colonies (Table 4-3). Some proliferation of Gut 4 may have also occurred at this concentration. Similar results of the stimulation of cell growth by low concentration of lytic peptides have also been reported (Jaynes et al., 1993; Borth et al., 2001).

4.3.2 Effect of transgenic anthuriums on populations of beneficial bacteria

Four species of beneficial bacteria originally isolated from the guttation fluid of two blight susceptible anthurium cultivars were inoculated onto transgenic and control anthuriums. These bacteria species were re-isolated from guttation fluid at 6 and 11 days post-inoculation. Bacterial counts from transgenic and control lines were compared to determine if anthuriums transgenic for the Shiva-1 peptide altered the bacteria populations.

Average counts, expressed as log CFU/ml guttation fluid, of the four species of beneficial bacteria at both testing dates (except Gut 5 at 11 days) were not significantly different ($P \leq 0.05$) between transgenic and control anthuriums for both cultivars (Table 4-4) despite differences in amount of Shiva-1 produced per line in clonal material (A. Kuehnle, unpublished).

Population counts of Gut 5 were significantly lower in MK 2-6 than ($Pr > F = 0.0275$) MK 1-2 on the 11^{th} day after inoculation (Table 4-4d). This is unusual because mean counts of Gut 5 from both transgenic lines did not differ from the control. This may be due to the lower number of replicate plants of MK 2-6 used in the experiment or experimental error.

In general, bacterial populations decreased by one order of magnitude from six

Table 4-3. Bacterial colony counts after 16-18 hrs. incubation with Shiva-1. Means with same letter in a column were not significantly different by Student-Newman-Kuels Test at $P \leq 0.05$. Note proliferation effect of Shiva-1 at 0.5 μM and 4 μM for Gut 6.

Shiva-1	Bacteria colony counts				
Conc. (μM)	Gut 3	Gut 4	Gut 5	Gut 6	Xcd Lux
0	159 b	196 ab	128 a	4 c	72 a
0.0625	125 b	183 ab	26 cd	12 c	-
0.125	144 b	191 ab	50 bc	5 c	64 a
0.25	151 b	249 a	65 b	6 c	40 b
0.5	201 a	262 a	49 bc	10 c	16 bc
1	277 a	165 b	2 d	106 a	6 c
2	10 c	0 c	0 d	39 c	0 c
4	0 c	0 c	0 d	69 b	0 c

Table 4-4. Bacterial counts re-isolated from guttation fluid of trangenic and control anthuriums. (A) 'Marian Seefurth' lines at 6 days post-inoculation, (B) 'Mauna Kea' lines at 6 days post-inoculation, (C) 'Marian Seefurth' lines at 6 days post-inoculation, and (D) 'Mauna Kea' lines at 11 days post-inoculation. Means with same letter in a column were not significantly different by Duncan-Waller test at $P \leq 0.05$.

	Bacterial counts (log CFU/ml)			
Plant line	Gut 3	Gut 4	Gut 5	Gut 6
MS 1-1	3.9 a	4.5 a	2.2 a	0.8
MS 1-5	3.7 a	3.9 a	2.7 a	0.6
MS C	3.6 a	3.8 a	1.9 a	0.8

A

	Bacterial counts (log CFU/ml)			
Plant line	Gut 3	Gut 4	Gut 5	Gut 6
MS 1-1	2.0 a	2.5 a	1.4 a	0.5 a
MS 1-5	3.4 a	4.8 a	1.7 a	2.0 a
MS C	2.6 a	2.8 a	1.6 a	1.8 a

C

	Bacterial counts (log CFU/ml)			
Plant line	Gut 3	Gut 4	Gut 5	Gut 6
MK 1-2	4.5 a	5.3 a	4.5 a	2.8
MK 2-6	5.6 a	6.7 a	5.9 a	2.7
MK C	5.3 a	6.2 a	5.0 a	1.9

B

	Bacterial counts (log CFU/ml)			
Plant line	Gut 3	Gut 4	Gut 5	Gut 6
MK 1-2	4.7 a	6.0 a	3.7 a	2.6 a
MK 2-6	2.8 a	4.1 a	1.7 b	1.4 a
MK C	3.6 a	4.6 a	2.6 b	1.7 a

D

days post-inoculation to twelve days post-inoculation (Table 4-5). Such decreases are to be expected in non-transgenic anthurium cultivars (Alvarez, unpublished data). Bacterial populations declined the most in MK 2-6 where fewer then on percent of the BCA cells were recovered eleven days post-inoculation with the exception of Gut 6 (Table 4-5). In contrast, MS 1-5 appeared to support the growth of several BCAs; populations of Gut 3, Gut 4, and Gut 6 increased by about two orders of magnitude in this line (Table 4-5). *Xcd* Lux also increased by two orders of magnitude from six to twelve days post-inoculation when inoculated onto control 'Marian Seefurth'. Counts of *Xcd* Lux from inoculated transformed plants are to be done in the future. The occurrence of increased and decreased population counts of the bacteria in control and transgenic plants illustrates that the transgenic status of the plant does not significantly decrease populations of the beneficial bacteria residing in the hydathodes.

4.4 DISCUSSION AND CONCLUSION

An in vitro bactericidal assay of different concentrations of Shiva-1 was tested on four beneficial bacteria and on one pathogen. Concentrations of Shiva-1 necessary for killing the beneficial bacteria and pathogen were similar to other antimicrobial peptides that generally kill bacteria (gram-positive and gram-negative) at concentrations ranging from 0.25 to 4 μg/ml (Hancock and Lehrer, 1998). Gut 6 was determined to be least susceptible to Shiva-1 with an apparent IC_{100} higher than 4 μM (16.96 μg/ml), the highest concentration used in this experiment. Shiva-1 at 4 μM was lethal to all other bacteria species tested. Gut 3 was determined the next least sensitive bacteria species with an

Table 4-5. Change in bacterial counts from 6 weeks post-inoculation to 11 weeks post inoculation.

		log of colony counts				
Lines	Assay day	Gut 3	Gut 4	Gut 5	Gut 6	*Xcd* Lux
MS 1-1						
	6	3.92	4.54	2.16	0.81	-
	11	2.38	3.05	1.73	0.6	-
	% cells remaining	3	3	37	62	-
MS 1-5						
	6	3.71	3.94	2.73	0.68	-
	11	4.27	6.04	2.1	2.49	-
	% cells remaining	263	12488	24	6337	-
MS C	6	4.45	4.48	2.32	0.95	3.60
	11	3.24	3.44	2.01	2.22	4.64
	% cells remaining	7	5	5	1744	997
MK 1-2						
	6	5.23	6.15	4.53	3.26	-
	11	4.65	5.94	3.73	2.59	-
	% cells remaining	26	62	16	22	-
MK 2-6	6	5.56	6.70	5.86	2.75	-
	11	2.8	4.08	1.75	1.14	-
	% cells remaining	< 1	< 1	< 1	2	-
MK C	6	5.28	6.28	4.91	1.94	-
	11	3.63	4.63	2.58	1.66	-
	% cells remaining	3	2	1	51	-

IC_{100} between 2-4 μM Shiva-1 (8.48-16.96 μg/ml). IC_{100} values for the other bacteria species (Gut 4, Gut 5, and *Xcd* Lux) ranged between 1-2 μM (4.24-8.48 μg/ml) Shiva-1. In F.C. Chen's assays (1993), 1.25 μg/ml of Shiva-10 was determined to inhibit growth of a similar pathogenic strain, *Xcd* 150, at a concentration of 385 CFU/ml.

In the subsequent experiment, effects of Shiva-1 produced by transgenic anthuriums were evaluated on the same bacterial species in planta. Anthuriums transgenic for the Shiva-1 peptide and control were inoculated with a mixture of four different beneficial species naturally present in leaves of field-grown anthuriums. Re-isolation of these bacteria from the plants guttation fluid determined no significant differences among the population of the four species of beneficial bacteria between transgenic lines and control anthuriums at 6 and 11 days post-inoculation.

Based on in vitro inhibition and proliferation of bacteria seen in guttation fluid, questions on transgene expression levels must be considered. Similar bacterial counts from transgenic and control plants may indicate transgenic plants were producing Shiva-1 at concentrations insufficient to alter the population of the beneficial bacteria and/or were not being transported to the target intercellular locations. These levels may still be adequate to control *Xcd*, which is more sensitive to Shiva-1.

Results may also support previous studies where lytic peptides failed to confirm resistance to pathogens (Mills et al., 1994). These studies suggest that the peptides do not retain their bactericidal activity in the target locations or are degraded by proteases within the intercellular fluid. Our experiments did not determine whether the Shiva-1 peptide was being directed to the leaf intercellular space (apoplast) and/or effectively released into the plants guttation fluid.

In theory, if the population of the beneficial bacteria is unaffected by transgenic plants and assuming the peptide is being produced and transported to the target locations within the plant, a two-pronged approach using transgenic plants primed with beneficial bacteria could provide enhanced and durable protection against anthurium blight disease for the five to seven years that plants are in production. The epiphytic nature of the beneficial bacteria can provide an initial defense against the invading pathogen (A. Alvarez, unpublished and Fukui et al., 1999ab). Their ability to prevent infection through the hydathodes and suppress the establishment of the pathogen in these areas may be the first step in attaining enhanced resistance to the anthurium bacterial blight. Added protection can be obtained from peptides produced by the anthuriums. Once the pathogen enters the intercellular spaces of the plant where beneficials are absent, peptides being excreted from the cells can target and kill the pathogen preventing further infection. However, further studies on the mechanism and the activity of Shiva-1 in the plant need to be conducted to fully support this theory.

On the basis of the presented data we have determined the ranges for 50 % and complete inhibition by Shiva-1 required to alter the population of four beneficial plant-associated bacteria and anthurium bacteria blight pathogen. Additional research is being conducted to determine transgene introgression and expression of the Shiva-1 peptide transcripts in the leaves of the plants. Results of these on-going experiments will provide a better understanding of the effects of anthuriums transformed with Shiva-1 on the population of beneficial bacteria naturally residing in anthurium leaves.

4.5 LITERATURE CITED

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APPENDIX A

MODIFIED MURASHIGE AND SKOOG MEDIUM (1 liter)

MS macro	50 ml
MS micro	10 ml
MS F (iron) stock*	5 ml
MS G (vitamins) stock**	2.5 ml
Myo-inositol (10 mg/ml)	10 ml
Coconut water	150 ml
Sucrose	20 g
Agar	7 g
pH	5.7-5.8

* MS F stock (250 ml), 1.835 g NaFe EDTA

** MS G stock (500 ml)

Nicotinic acid	0.05 g
Pyridoxin HCl	0.05 g
Thiamine HCl	0.01 g
Glycine	0.2 g

APPENDIX B

YEAST DEXTROSE CALCIUM CARBONATE (YDC) MEDIUM (1 liter)

Yeast extract	10 g
Dextrose	20 g
Calcium carbonate	20 g
Agar	17 g

APPENDIX C

TRIPHENYLTETRAZOLIUM CHLORIDE (TTC) MEDIUM
(modified by Norman and Alvarez, 1989)

Peptone	10 g
Dextrose	5 g
2,3,5-Triphenyltetrazolium chloride	1 mg
Difco Bacto agar	17 g

APPENDIX D

TRIPHENYLTETRAZOLIUM CHLORIDE (TTC) MEDIUM WITH ADDITION OF ANTIBIOTICS (to 1 liter) FOR SELECTIVE GROWTH OF DIFFERENT BACTERIAL SPECIES

GUT 3	50 μg/ml rifampicin, 200 μg/ml streptomycin, and 100 μg/ml cycloheximide
GUT 4	5μg/ml polymyxin B, 10μg/ml tetracycline, and 100 μg/ml cycloheximide
GUT 5	50μg/ml kanamycin, 25 μg/ml trimethoprim, 10 μg/ml TTC, and 100 μg/ml cycloheximide
GUT 6	50 μg/ml rifampicin, and 100 μg/ml cycloheximide
XCD LUX	50 μg/ml rifampicin, 10μg/ml tetracycline, and 100 μg/ml cycloheximide

APPENDIX E

DISEASE SYMPTOMS ASSESSED VISUALLY

Line and rep.	Leaves infected	No. of infection sites
MS 1-2		
1	75% (3/4)	7
2	50% (2/4)	2
3	67% (2/3)	3
4	25% (1/4)	2
5	50% (2/4)	2
6	25% (1/4)	2
7	50% (2/4)	4
8	50% (2/4)	4
9	25% (1/4)	2
10	25% (1/4)	3
MS 1-4		
1	50% (2/4)	4
2	25% (1/4)	4
3	25% (1/4)	1
4	50% (2/4)	3
5	67% (2/3)	3
6	25% (1/4)	2
7	25% (1/4)	1
8	33% (1/3)	2
'Rudolph'		
1	100% (4/4)	not determined
2	100% (4/4)	"
3	100% (4/4)	"
4	100% (4/4)	"
5	100% (4/4)	"
6	100% (4/4)	"
7	75% (3/4)	"

APPENDIX E (cont.)

DISEASE SYMPTOMS ASSESSED VISUALLY

Line and rep.	Leaves infected	No. of infection sites
UH 712 1-1		
1	0% (0/4)	0
2	50% (2/4)	5
3	25% (1/4)	4
4	0% (0/4)	0
5	25% (1/4)	5
6	0% (0/4)	0
7	0% (0/4)	0
8	25% (1/4)	1
9	25% (1/4)	3
10	50% (2/4)	5
UH 712 1-9		
1	50% (2/4)	2
2	0% (0/4)	0
3	33% (1/3)	4
4	0% (0/4)	0
5	0% (0/4)	0
6	50% (2/4)	4
7	25% (1/4)	2
8	0% (0/4)	0
9	0% (0/4)	0
10	0% (0/4)	0
UH 712 1-16		
1	100% (4/4)	12
2	50% (2/4)	4
3	100% (4/4)	18
4	25% (1/4)	2
5	100% (4/4)	not determined
6	100% (4/4)	"
7	100% (4/4)	"
8	100% (4/4)	"
9	100% (4/4)	"

APPENDIX E (cont.)

DISEASE SYMPTOMS ASSESSED VISUALLY

Line and rep.	Leaves infected	No. of infection sites
UH 712 C		
1	25% (1/4)	3
2	75% (3/4)	6
3	0% (0/4)	0
4	0% (0/4)	0
5	25% (1/4)	4
6	33% (1/3)	1
7	25% (1/4)	2
8	0% (0/4)	0
9	0% (0/4)	0
10	50% (2/4)	3